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CONDITION OF FERTILIZER POTASH RESIDUES IN HAGERSTOWN SILTY LOAM SOIL[1]

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INTRODUCTION

It has long been known that when solutions of potassium salts are brought into contact with clayey or loamy soils, the potassium is quite rapidly removed from solution by the soil, with or without replacement in the solution by other basic elements in chemically equivalent amounts; also, that by immediate washing of the soils thus treated a large part of the potassium they have acquired from the potassium-salt solution can be recovered from the soil.

It has furthermore been shown by numerous investigations that, when the potassium salt is introduced into a clay or loam soil in a solid, such as the potash salt of a commercial fertilizer, the potassium is quite promptly "fixed," or united with the soil solids and made stationary at the point of introduction; that the downward movement of the potassium into the subsoil is relatively slight, and that the loss by drainage is small. On the other hand, in a sandy soil the drainage loss may be large (*2*).[2]

The testimony concerning the usefulness to crops of the potash thus fixed in the soil, the crop increases obtained in many instances by potash fertilization, and also the quantity of potassium taken up by the crop to which the fertilizer has been applied is that, whether a crop increase follows the potassium dressing or not, the crop is, in the majority of cases adequately studied, richer in potassium than the crop grown simultaneously upon the same soil without such fertilization. In general, the amount of potassium taken up by the crop fertilized with potash as contrasted with that not so fertilized is proportionally much greater than the increase in the total potassium supply of the soil due to the potassium dressing supplied.

[1] Approved for publication, by R. L. Watts, Dean and Director, Pennsylvania Agricultural Experiment Station.

[2] Reference is made by number (italic) to "Literature cited," p. 81.

There is, indeed, much evidence that the crop fertilized never takes off potassium in amount equivalent to that contained in the usual fertilizer dressing; nor, very often, half as much. The agricultural value of the residual fertilizer potash is consequently a point of much economic importance, especially in view of present prices for fertilizer potash.

The final criterion of such value is, of course, in the crop yields and their potassium content noted at successive periods after potash fertilizing. There are of record few long-continued field or pot studies upon this point.

Chemical methods of soil examination are doubtless not strictly comparable with biological methods for determining the degree of "availability" of a plant food present in the soil, or for determining the quantity of such material present in different degrees of availability. They do serve, however, to determine the existence of differences in the condition of solubility of such plant food, and in a roughly quantitative way, the amounts present in the conditions contrasted.

PREVIOUS WORK

Numerous chemical studies of this sort have been made upon soils representing comparatively brief periods of fertilizer treatment with collectively small amounts of added fertilizer constituents. Few such studies represent, however, long periods of contrasted treatments definitely maintained. Of these, that reported by Dyer (3) from the examination of the barley soils of the Hoos Field, Rothamsted, is in many respects the most important. The treatment contrasted had continued for 38 years, and not only the amount and composition of the fertilizers but also the crop yields and the ash content and composition of the crops removed, were known for two of the plots, soils from which were analyzed. Of these two plots, No. 2A had received no fertilizer potash, No. 4A, 4,100 pounds. The potash had not largely increased the crop yields, but had increased the potash content of the barley; so that, while the 38-year yields from plot 2A contained 984 pounds of potash, those from plot 4A contained 2,057 pounds. The net residuum of fertilizer potash on plot 4A was, therefore, 2,043 pounds; whereas, plot 2A had lost 984 pounds in the crops, making a net contrasted difference between the two plots of 3,027 pounds. The results of the chemical examinations of the two soils at the end of the 38-year period with respect to potash are summarized as follows:

Constituent.	Potash (pounds per acre).			Ratio, 2A:4A.
	Plot 2A.	Plot 4A.	Difference.	
Total potash (modification of Smith method).	36,376	43,301	6,925	1: 1.19
Potash dissolved by strong hydrochloric acid (method not accurately described).....	6,269	8,242	1,973	1: 1.31
Potash dissolved by 1 per cent citric acid acting for two weeks at room temperature.	57	753	696	1:13.21

Moreover, in five groups of plots from the same field, each with two plots receiving no potash and two that were periodically dressed with potash fertilizers, the soils of the potash-dressed pair invariably contained more total potash than those of the nonpotash pair; with a single exception, more potash soluble in hydrochloric acid; and in all cases much more potash soluble in 1 per cent citric acid, the average ratio for the nonpotash to potash-treated soils in this last case averaging 1 to 9.

PENNSYLVANIA STUDIES

The soils used for the present study represent plots 1 and 4, Tier II, of the General Fertilizer Experiments of the Pennsylvania Experiment Station, which have been maintained continuously since 1881. All the plots of Tier II were cultivated and cropped alike, the land being kept under the common Pennsylvania 4-course rotation of corn (*Zea mays*), oats (*Avena sativa*), wheat (*Triticum aestivum*), and grass (mixed timothy (*Phleum pratense*) and medium red clover (*Trifolium pratense*). Plot 1 received no dressings of any kind during this experiment. Plot 4 was dressed biennially, for corn and wheat, with 200 pounds of muriate of potash, so that, beginning with 1881, it had received 18 such dressings, equivalent to 1,800 pounds of potash (K_2O) up to the time of the sampling in 1916, when the land was in oat stubble. The latest of these dressings had been applied 14 months prior to the sampling.

From 1868 to 1881 the plots were used for certain cultivation experiments, plot 1 being plowed with a common plow for corn and wheat, plot 4 with a subsoil plow for corn, and a Michigan plow for wheat. Both plots were, however, cropped alike and were also fertilized alike and not at all heavily. Prior to 1868 the land was under general farm cultivation and cropping, the two plots forming part of the same field and doubtless having had the same farm history.

The weight of the acre 7-inch surface layers of the two plots was determined[1] at 22 points uniformly distributed over each plot, with details of method and result that have been elsewhere reported.[2] The air-dry (not oven-dry) weights of the respective surface 7-inch layers were: Plot 1, 2,091,662 pounds to the acre; plot 4, 2,036,449 pounds to the acre. The probable error of these determinations was less than 10,000 pounds to the acre.

The 22 subsamples obtained in two independent series from each plot in the course of the acre-weight determinations were supplemented by 80 other subsamples, likewise divided into two independent series for each plot, obtained by means of a soil auger. Both sets of subsamples were prepared in the same manner, by air-drying and sifting all of each

[1] All quantitative determinations reported were, unless otherwise specifically credited, made by Mr. ERB under the supervision of the senior author.

[2] FREAR, WILLIAM, and ERB, E. S. EXCAVATION METHOD FOR DETERMINING THE APPARENT SPECIFIC GRAVITY OF SOILS. To be published in Proc. Assoc. Off. Agr. Chem. 1917.

subsample successively through a 4-mm. and a 1/20-inch (1.27 mm.) sieve. The fine soils obtained from the latter sifting were in each case composited so as to represent the four series of subsamples obtained from each plot, and the composites were then submitted to partial analysis. The results of this study of sampling methods have also been elsewhere reported in detail.[1]

The net results for the proportion and amount of fine soil (passing a 1/20-inch sieve) in the surface of the two plots, which contain a good many cherty fragments, were:

	Plot 1.	Plot 4.
Fine soil (per cent of air-dry sample).....	90.39	91.59
Fine soil (pounds to the acre 7 inches).....	1,890,644	1,865,947

The analyses of the several series composites from the same plot show composition differences somewhat greater than appear in duplicate analyses of the same composite. These series differences are rarely more than double the differences shown by duplicate determinations upon the same composite. They are not of such magnitude as to approach the differences exhibited by the analytical figures, either detailed or average, for the respective plots. For this reason the several sets of detailed analyses are not presented in full in the present account; but, when the repetitions of a determination upon composites from the same plot are sufficiently numerous, they have been used, by application of the Gauss formula, to calculate the probable error of the determination.

Because of the exceptional care employed in taking, preparing, and compositing the samples, it is believed that the materials used for this study are in an unusual degree representative of the soil areas from which they were obtained.

All analytical results stated in this paper are expressed in terms of the air-dry soil, without recalculation to a water-free basis. Determinations of hygroscopic moisture made when the analytical work was begun showed a range of 0.785 to 1.088 per cent for the eight series composites, with plot averages of 0.816 and 0.829 per cent, respectively. Another set of such determinations made about the close of the analytical work showed an average increase of only 0.05 per cent of moisture in the soils.

TOTAL POTASH

Portions of the series composites were reduced to an impalpable powder by grinding in an electric mortar mill of agate. The total potash content in each was determined in duplicate by the J. Lawrence Smith method as described by Washington (*9, p. 129*). The final weights of potassium platinichlorid were ascertained by first weighing the dried precipitate in a Gooch crucible, then washing out the soluble

[1] FREAR, WILLIAM, and ERB, E. S. A STUDY IN SOIL SAMPLING. To be published in Proc. Assoc. Off. Agr. Chem. 1917.

salt with hot water, and again weighing, after drying at 100° C., the crucible with its felt and any residual, insoluble impurities, of which a small amount always appeared. The difference between the two weights thus obtained was counted as the weight of potash salt. This procedure was used in all other potash determinations here reported.

The results of eight determinations of total potash for each plot were:

	Per cent.
Plot 1, untreated	3.821 ± 0.0240
Plot 4, potash dressed	3.543 ± .0134

The fact, curious at first blush, appears that the potash-treated plot contains at this time actually less potash than the untreated plot. The probable explanation of this condition is found in the rather high, natural variability in composition of the soils of the general series of plots, which has been established by other studies of these soils (*4, p. 187*).

POTASH SOLUBLE IN HOT, STRONG HYDROCHLORIC ACID

The method followed was substantially the old official method [1] as prescribed for cases in which only the alkalis are to be determined—that is, 10 gm. of the air-dry fine soil in its natural condition of subdivision were exposed for 10 hours on steam cups or in water bath to the solvent action of 100 cc. of hydrochloric acid (1.115 sp. gr.), with hourly shaking. The heavy metals and alkaline earths were thrown out of the solution by barium hydrate, and the excess of this precipitant was removed from the filtrate as oxalate. Each of the precipitates was washed with from 600 to 700 cc. of warm water to insure the complete recovery of the alkalis therefrom. All subsequent details of procedure designed for determining the sodium in the solution were omitted.

It is a matter of common experience that, of all the more abundant soil elements, potassium reacts most sensitively to the conditions of acid solution. Hilgard (*6, p. 342*) has emphasized this fact, and Frear and White (*4, p. 187*) have demonstrated it for the potash in the soils of the general fertilizer tract.

The temperature condition is especially influential upon the rate of solution for this constituent. In the first set of these determinations the dissolving flasks were heated on steam cups. While the solutions obtained gave in most cases fairly concordant duplicates, in others the differences between duplicates were equal to fully 20 per cent of the potash dissolved. These differences are attributed to inequalities of the temperatures maintained on the different cups, and in part also to the differences in the agitation of the liquids on different cups.

Another set of determinations was made later, in which the dissolving flasks, provided as in the former set with reflux condensers, were sus-

[1] REPORT OF COMMITTEE ON EDITING TENTATIVE AND OFFICIAL METHODS OF ANALYSIS [ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS], p. 24–25. 1916.

pended in a water bath to such depth that the level of water in the bath was somewhat higher than that of the acid in the flasks. The water was at boiling temperature (about 99° C.) when the flasks were introduced, and was so maintained during the solution process. The water level in the bath was maintained by frequent, small additions of water, and the flasks given a rotary shaking each hour. The bumping of the bath insured, however, a constant agitation of the soil patricles in the acid solvent. The maximum difference between duplicates observed when this solution method was followed was equivalent to no more than 3 per cent of the potash dissolved.

Plot and treatment.	On steam cups.	In water bath.
	Per cent.	*Per cent.*
Plot 1, untreated	[a] 0.2725±0.00346	[c] 0.3687
Plot 4, dressed with potash	[b] .3110± .005	[d] .4072±.00146

[a] 10 determinations.
[b] 16 determinations.
[c] Duplicate determinations.
[d] 6 determinations.

The water-bath treatment gave in each case about one-third more potash than was obtained by heating in the steam cups. Because the prescribed conditions were more certainly maintained by use of the water bath, and also because the results thus obtained are the less variable, these results are accepted as the better representing the method.

POTASH SOLUBLE IN WARM N/5 HYDROCHLORIC ACID

For this determination 100 gm. of the fine soil was used in its natural condition of subdivision, with 1,000 cc. of *N*/5 hydrochloric acid. The dissolving flasks were maintained at a temperature of 40° C. in an electric oven for five hours, and were well shaken at hourly intervals during that period. The solutions were then promptly separated from the undissolved soil by filtration. A 500-cc. aliquot of the filtrate was evaporated down for analysis. The details of analysis of the solutions were as previously described for the solutions otherwise obtained.

There were eight determinations for each plot, with results as follows.

	Percentage of potash (K_2O).
Plot 1, untreated	0.0143±0.00017
Plot 4, dressed with potash	.0301± .00019

POTASH SOLUBLE IN DISTILLED WATER

Determinations were attempted by three different methods of solution, with different ratios of soil to solvent, all at moderate temperatures, of the potash that could be dissolved by distilled water from the respective fine soils in their natural state of subdivision.

FLASK METHOD.—A 10-gm. portion of the soil was heated with 1,000 cc. of freshly boiled and cooled distilled water in an electric oven at 40° C. for five hours, with vigorous shaking at hourly intervals. At the end of the heating period the solution was filtered quickly away from the undissolved soil, and freed from suspended silt and clay by repeated filtration. The entire filtrate, after it had been freed from visible suspended matter, was concentrated by evaporation. To destroy the dissolved organic matter, the solution was evaporated to dryness, ignited, and the residue taken up with dilute acid. The determinations of potash were made gravimetrically as in the preceding solutions, but with highly variable results. Although the results from the respective soils, obtained by five repetitions for plot 1 and eight for plot 4, were—

	Percentage of potash (K_2O).
Plot 1, untreated	0.0032 ± 0.0003
Plot 4, dressed with potash	.0049 ± .0006

the ranges of variation were, for plot 1, from 15 to 46 parts of potash per million of the soil, and for plot 4, from 17 to 75 p. p. m.

PERCOLATION METHOD.—For this method 50-gm. composites for each plot were gradually filled into ⅞-inch percolation tubes, closed below by means of a double paper filter reenforced by a linen filter firmly bound around the lower end of the tube. The soil was lightly tamped as it was filled into the tubes, so thot in each case the dry soil column had a final depth of 3¾ inches. The distilled water was delivered from a Bunsen bottle suspended over the tube at such rate as to maintain a 1-inch water head above the level of the light, acid-extracted asbestos wad placed over the soil to secure uniform entry of the liquid into the soil column and to prevent spattering and superficial packing of the soil. Four such tubes containing duplicate portions of the two soils were supported side by side at room temperature (about 21° C.), under like evaporation conditions, and the percolation was continued until 500 cc. of percolate had been obtained from each. The times required for the percolation differed somewhat.

The percolates were filtered until free from clay, and potash was then determined as in the preceding solutions, with the following results:

Plot.	Treatment.	Set.	Percolation time.	Potash.	
				Found.	Average.
			Days.	*Per cent.*	*Per cent.*
1	Untreated	a	4	0.0048	0.00455
1	do	b	4	.0043	
4	Dressed with potash	a	4	.0083	.00800
4	do	b	6	.0077	

CLAY SEPARATION WATER.—For a purpose stated later, a mechanical separation of the clay from these soils was made in the following manner: Six hundred gm. of the air-dry fine soil was shaken with about 2 liters of distilled water in a rotary shaker for eight hours. After the suspensions thus obtained had settled overnight, the clayey liquor was carefully siphoned off. The soil residues were then thoroughly agitated with a fresh portion (1.5 to 2 liters) of distilled water, then allowed to stand until the particles of more than 0.005 mm. in diameter had settled out, when the new clay suspension was siphoned off. This washing process was repeated for 10 days. Even then not all the clay had been removed from the coarser soil members. The volumes of the combined washings were, for plot 1 soil, 16,710 cc.; for plot 4, 18,530 cc.

To remove the clays from these suspensions they were allowed to stand for two days, by the end of which five-sixths of the clay had settled out. The overlying liquors were carefully drawn off, and the clay still remaining in suspension in the liquor was almost completely removed by flocculation. The flocculating material first used was ammonium chlorid. This was added in 5-gm. portions previously dissolved in a little distilled water, and thoroughly stirred through the liquor.

In the case of the suspension from plot 1 soil, flocculation began shortly after the addition of two portions, or 10 gm., of the flocculant; but three portions, or 15 gm., caused no appearance of flocculation in the suspension from plot-4 soil. This liquid had the appearance which slight alkalinity gives to clay suspensions, although rather sensitive pink litmus gave no alkaline reaction when moistened with it. The addition was tried, therefore, of concentrated hydrochloric acid, drop by drop, with stirring after each drop had been added. Upon the introduction of the tenth drop, flocculation began rather sharply, so that the addition of the acid was discontinued.

The liquors drained off from these flocculated clays were still faintly cloudy, and separated a little clay on long standing.

Of the washings from plot 1, 14,530 cc., and of those from plot 4, 13,030 cc. were withdrawn, filtered, evaporated to dryness, and the evaporation residue ignited to get rid of organic matters and ammonium salt. The ignited residues were moistened with acid, taken up with hot water, filtered, and subjected to analysis for potash by the method already described.

The quantities of potash found in the total wash waters were:

	Percentage of soil.
Plot 1, untreated	0.0038
Plot 4, dressed with potash	.0087

In view of the differences in method of treatment, in proportion of soil to water, and in duration of the exposure of soil to solvent, it is remarkable that the quantities of potash removed by water were in all cases so nearly the same for the respective soil samples.

POTASH SOLUBLE IN CARBONATED WATER

The quantities of soils used and arrangement of apparatus were substantially the same as those in the percolation with distilled water. The principal modifications consisted in (1) the delivery of water charged with carbon dioxid at air pressure and room temperature by means of a continuous current of the gas passed through the water in the Bunsen bottle; and (2) the closing of the top of the percolator tube by means of a one-hole rubber stopper, to prevent loss of the gas from the apparatus at that point.

The percolations were continued until in each case 500 cc. of percolate had been extracted. The linen filter was washed in both percolation tests with a spray of water to remove any soluble film that might have been deposited by evaporation of the percolate. The washings were added to the latter.

The usual analytical procedure was followed, with these results, in terms of the air-dry soil:

Plot.	Treatment.	Set.	Time of percolation.	Potash (K_2O).	
				Found.	Average.
			Days.	*Per cent.*	*Per cent.*
1	Untreated	a	5	0.0088	0.0076
1	do	b	2	.0064	
4	Dressed with potash	a	4	.0162	.0140
4	do	b	3	.0123	

While the percolates which passed more slowly through the soil contained more potash than those which passed quickly, the increase was not proportional to the time of percolation for either soil.

POTASH SOLUBLE IN AMMONIUM-CHLORID SOLUTION

One hundred gm. of the air-dry fine soil in its natural state of subdivision were heated with 1,000 cc. of the neutral-salt solution for five hours in an electric oven at 40° C. with hourly shaking. At the end of the heating period the liquors were immediately filtered off from the undissolved soils, and subjected to the analytical procedure previously described.

To find a satisfactorily active solution concentration of the ammonium chlorid without too greatly increasing the amount of this salt that must later be removed in the analytical process, the effects of solutions containing 17.6 and 50 gm., respectively, of the salt in 1 liter of distilled water (approximately $N/3$ and normal solutions) were compared

with that of distilled water. The results, in terms of air-dry soil, obtained with a sample from plot 4, were:

Solvent.	Set.	Potash.		
		Found.	Average.	Excess over water effect.
		Per cent.	*Per cent.*	*Per cent.*
Distilled water	a	0.0075	0.0073	.....
	b	.0070		
N/3 ammonium-chlorid solution	a	.0194	.0190	0.0177
	b	.0186		
Normal ammonium-chlorid solution	a	.0220	.0216	.0143
	b	.0212		

This shows that, under the conditions maintained, the *N*/3 solution had about 2.7 times the solvent effect of water alone; and the normal solution about 3.1 times that of water. In other words, trebling the concentration of the salt solution increased its solvent effect little more than one-eighth.

Hence, as the *N*/3 solution introduced no more ammonium chlorid than could be handled with a fair degree of convenience in the analytical operations, a solution of that concentration (17.6 gm. of salt to 1,000 cc. of water) was used in the remaining studies with the solvent.

The average results for nine samples of each soil were, in terms of the air-dry soil and corrected for blank:

	Percentage of potash (K_2O).
Plot 1, untreated	0.00936±0.00012
Plot 4, dressed with potash	.01872± .00039

In order to ascertain the relative solvent effect of a second extraction with this solution after two of the treatments, one for each plot, included in the above averages, the once-extracted soil residues were carefully washed with a little water to remove the excess of the salt solution, dried on the filter, and again subjected, as before, to the action of 1 liter of the fresh salt solution. The results obtained in the respective extractions were:

	Percentage of potash (K_2O).
Plot 1, first extraction	0.01002
Plot 1, second extraction	.00198
Plot 4, first extraction	.02210
Plot 4, second extraction	.00208

The interesting fact appears that the amounts of potash removed in the second extraction were almost identical for both soils, and in both cases were much less than the quantities removed by the first extraction. In order to check this finding, a second portion of plot-4 soil was

thus extracted a second time, with a recovery (net) of 0.00190 per cent of potash in the second extract.

It happens that the quantity of potash removed by the two extractions of the soil from plot 4 was practically the same as the amount removed by a single extraction with ammonium-chlorid solution of normal concentration.

Summary of fine-soil potash solubilities

Treatment of potash.	In terms of air-dry fine soil.		In terms of total potash.	
	Plot 1.	Plot 4.	Plot 1.	Plot 4.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*
Total	3.821	3.543	100.000	100.000
Soluble in hot (1.115 sp. gr.) hydrochloric acid, 10 hours, 1:10	.3687	.4072	9.649	11.493
Soluble in warm (40° C.) *N*/5 hydrochloric acid, 5 hours, 1:10	.0143	.0301	.374	.850
Soluble in distilled water:				
Flask method, 5 hours 40° C., 1:10	.0032	.0049	.084	.138
Flask method, highest results	.0046	.0075	.120	.212
Percolation method, 21° C., 1:10, 4 to 6 days	.0045	.0080	.118	.226
In water from clay washing	.0038	.0087	.099	.246
Soluble in carbonated water by percolation, 21° C., 1:10, 2 to 5 days	.0076	.0140	.199	.395
Soluble in *N*/3 ammonium-chlorid solution, 5 hours, 40° C., 1:10:				
First extraction	.0094	.0187	.246	.528
Second extraction	.0020	.0021	.052	.059

In general, only about one-tenth of the potash of these soils can be extracted from the fine soil in its natural state of subdivision by strong, hot hydrochloric acid acting at the temperature of boiling water for a period of 10 hours, when 10 cc. of the acid are used for each gram of the soil.

When the conditions of solution are closely maintained, the potash of the potash-fertilized plot is almost one-fifth more soluble in strong, hot acid than that from the untreated plot.

When mild solvents are used to extract the potash, the differences in the condition of this element in the two soils are much more pronounced. The solubility of the potash in the potash-fertilized plot is from 1.5 to 2.5 times greater than in the untreated plot.

The similarity of the ratios between the percentages of potash dissolved from the two soils by the respective mild solvents, distinctly suggests identity in the nature of the materials acted upon in the soils by these solvents. On assuming that water and its solutions of carbon dioxid and of ammonium chlorid act only upon those potassium compounds of the soil that weak hydrochlorid acid can attack, and expressing the

amounts of potash dissolved by the former as percentages of the entire amount dissolved by the *N*/5 acid, and in this comparison taking as typical of the water action the results obtained by percolation, because they are internally the most consistent, the percentages thus computed are:

Treatment of potash.	In terms of potash soluble in *N*/5 hydrochloric acid.	
	Plot 1.	Plot 4.
Dissolved by—	*Per cent.*	*Per cent.*
Distilled water (by percolation)	31.6	26.6
Carbonated water (by percolation)	53.2	46.5
Ammonium-chlorid solution, *N*/3	65.5	62.1

There appears a very close similarity between these percentages for the two soils. The figures for plot 4 lag somewhat behind those for plot 1, but probably no more than should be expected when the slow solution rate here controlling and the much larger amount of soluble potash in plot 4 are considered. In other words, there is here a close parallelism between the solvent effects with regard to potash of the *N*/5 hydrochloric acid and of the other weak solvents.

We may infer from these facts of chemical behavior that the residues from potash fertilization remain, at least in considerable measure, in a state of availability in the surface soil to which the fertilizer was applied. The final arbiter of availability is the plant itself. Certain plant evidence in our possession with respect to the availability of the potash in the soils under the treatments here contrasted will be presented later in this paper.

POTASH OF THE CLAYS AND NONCLAYS OF THE TWO SOILS

With the purpose of examining the condition of the potash in the two soils from another viewpoint, the clays were separated from the nonclays by the method of sedimentation in distilled water. The details of the process are given in the paragraphs dealing with the water solubility of the potash.

The clays and nonclays from each plot were assembled, with slight mechanical losses, on containers and filters, air-dried, and weighed.

The net results of the separations were:

Group.	Plot 1, untreated.		Plot 4, potash-dressed.	
	Weight.	Per cent.	Weight.	Per cent.
	Gm.		*Gm.*	
Clay, air-dry	70	11.67	66	11.00
Nonclay, air-dry	511	85.17	517	86.17
Solution and mechanical losses	19	3.16	17	2.83
Total	600	100.00	600	100.00

For a check upon the accuracy of the foregoing results obtained by the separatory treatment of relatively large quantities of soil, mechanical analyses of grand composites of the series composites for the respective plots were made by Mr. Walter Thomas, of this laboratory, using the Bureau of Soils methods and types of apparatus, upon 6-gm. portions of the fine soils. His results were:

Group.	Diameters.	Plot 1.	Plot 4.
	Millimeters.	*Per cent.*	*Per cent.*
Fine gravel	2 –1	.16	.33
Coarse sand	1 – .5	2.28	2.81
Medium sand	.5 – .25	2.86	2.90
Fine sand	.25 – .1	.56	.70
Very fine sand	.1 – .05	14.90	14.05
Silt	.05 – .005	63.50	63.46
Clay	.005– .0	10.08	10.24
Ignition loss		5.65	6.06
		99.99	100.55

These percentages differ somewhat from those reported earlier by Brown and Skinner (*1*, *p. 30*) for the surface soils of these plots, who found in brief:

Group.	Plot 1.	Plot 4
	Per cent.	*Per cent.*
Fine gravels and sands	11.9	15.9
Silt	73.6	69.5
Clay	13.9	13.9
Total	99.4	99.3

That is, they found less sand and more silt and clay, although their disregard of ignition losses somewhat increased their percentages.

In general, the figures obtained by the sedimentation of 600-gm. portions of the soil are essentially confirmed by the mechanical analyses.

The two soils are practically alike in their proportions of the mechanical components. The observed difference in the apparent specific gravity of the soils is probably the consequence chiefly of some unlikeness in the arrangement of the constituent soil grains.

THE CLAYS

The characters of the separated "clays" were distinctly claylike. When moist, they had a putty-like consistence; and, when dried, were hard and broke with a gluelike fracture. Microscopic examinations of the suspensions before the final sedimentation showed that most of the particles, visible under a one-twelfth objective, had diameters less than 0.0025 mm.

Portions of the clays were analyzed by the J. Lawrence Smith method, with these results, in terms of the amounts of clay:

	Percentage of total potash in clays.
Plot 1, untreated	3.310
Plot 4, dressed with potash	3.137

The clay from plot 4, as with the entire fine soil, contained less potash than the corresponding fraction of the plot 1 fine soil. In each case, however, the clay contained a less percentage of potash than appeared in the total fine soil.

THE NONCLAYS

The total potash in the washed nonclays was not determined directly. Computed by difference, the percentages are, in terms of the amounts of nonclays:

	Percentage of potash in nonclays.
Plot 1, untreated	4.027
Plot 4, dressed with potash	3.701

SOLUBILITIES OF THE POTASH OF THE WASHED CLAYS AND NONCLAYS IN *N*/3 AMMONIUM-CHLORID SOLUTION

The washed clays and nonclays thus separated and air-dried were treated in 50-gm. portions with 500 cc. of *N*/3 ammonium-chlorid solution for five hours at 40° C., with hourly shaking, and the resultant solutions were immediately filtered off and analyzed for potash. The results are stated below in terms of the total weight of clays and nonclays, respectively:

Potash dissolved from—	Plot 1, per cent.	Plot 4, per cent.
Clay, washed	0.0352	0.0408
Nonclay, washed	.0041	.0050

We may safely conclude from these studies that, although the clays are not so rich as the nonclays in total potash, weight for weight, they yield to weak solvents from six to eight times as much of this element during a short period of time. It is also clear that, while both fractions of the potash-dressed soil are richer in soluble potash than the corresponding fractions from the unfertilized soil, the clays differ in this particular much more than the nonclays.

The percentages of the total potash in these washed clays and nonclays that are removed by the ammonium-chlorid solution are:

Potash dissolved from—	Plot 1, per cent.	Plot 4, per cent.
Clay, washed	0.761	1.306
Nonclay, washed	.102	.132

That is, whether the amount dissolved be expressed in terms of the respective soil fractions or of their total potash contents, the solubility of the clay potash is much greater than that of the nonclay.

RELATION OF AMOUNTS OF POTASH DISSOLVED FROM CLAY AND NONCLAY TO THE COLLECTIVE SURFACE AREAS OF THEIR PARTICLES

Since one of the important factors determining the quantity of a given solid that can be dissolved by a given solvent is the amount of surface which the solid exposes to the liquid, it is worth while to attempt an approximation of the surface areas exposed to the solvent by the clays and nonclays of these soils, and to the amount of potash dissolved from unit areas respectively exposed to the solvent.

In the following tabular statement are given the areas, in square meters, of the clay and nonclay fractions in 100 gm. of the respective air-dry soils, as calculated by the conventional method (7, *p. 118*), using the formula

$$\text{Surface area} = \frac{6 \times w}{d \times 2.65}$$

in which "*w*" is the weight in grams of the group of soil particles for which the surface area is to be computed; "*d*," the mean diameter, in centimeters, of the particles of the group; and "2.65" the assumed specific gravity. The areas stated for the nonclays are the sums of those separately computed for the particles of each class as to fineness, other than the clays. Owing to the practical identity in mechanical composition of the soils of the two plots, the same percentages of mechanical composition were used in the calculations for both soils. With these areas (expressed in square meters in the table) are given the corresponding quantities in milligrams of the potash dissolved by the ammonium-chlorid solution from 1 square meter of the particle surface of the respective fractions.

If we accept without qualification these results of the conventional computation of the surface areas of the respective groups of soil particles,

we are faced with the apparent fact that the potash of the clay is much less soluble, in proportion to the quantity directly exposed to the solvent, than that of the nonclay. This would tend to negative, at least for these two soils, the widely held theory that the soil particles are covered with a fairly homogeneous celloid coating from which weak solvents take up most of the materials they gain from the soil in a short time after they begin to act upon it; or else, it must be assumed that such coatings are much thicker on the nonclay than on the clay particles.

Item.	Clay.	Nonclay.
Surface areas of fractions in 100 gm. of soil.....square meters..	9.1	5.7
Potash (milligrams) dissolved by *N*/3 ammonium-chlorid solution from 1 square meter of particle surface:		
Plot 1, unfertilized.....	.28	.61
Plot 4, dressed with potash.....	.45	.74

It is here needful, however, to qualify our acceptance of these conventional estimates of the surface areas of the particles acted upon.

It was noted in an earlier paragraph, that the larger proportion of the clay particles, when their watery suspensions were examined microscopically, showed diameters less than 0.0025 mm., the mean diameter for the clay group. The effect of the average diameter being in these cases less than the mean diameter for the clay group is, of course, to increase, possibly in quite large degree, the corresponding surface areas and to diminish correspondingly the quantity of potash dissolved from a unit area.

On the other hand, the portions of the washed clays and nonclays submitted to solvent action had been air-dried after separation by sedimentation. A portion of each of the air-dried clays was shaken up with water to see whether the drying had resulted in any flocculation or cementing of the particles. It was found difficult to get all the clay back into suspension, even with the aid of a little ammonia added to deflocculate. Still, the proportion of the dry clay not readily taken back into suspension was relatively small. Another condition, possibly much more highly determining in its effect, is found in the fact that ammonium-chlorid solution itself acts as a flocculant, so that a free exposure of the surfaces of the clay particles is impossible in its presence.

POTASH IN CROPS HARVESTED FROM THE UNTREATED AND POTASH-DRESSED LANDS

No studies of the potash content of the crops from plots 1 and 4 of Tier II were made in connection with the present investigations. There are available, however, two sets of analyses made of crops harvested at different times from the plots of corresponding numbers and history

belonging to Tiers III and IV, of the same General Fertilizer Experiments. The former of these sets of analyses was made by Pingree (*8*), using the oat crop harvested in 1904 from Tier IV. The second set is composed of unpublished analyses made by senior students of the Department of Agricultural Chemistry of The Pennsylvania State College under the direction of Dr. C. W. Stoddart, who has kindly permitted the use of the results obtained. This second set of analyses represents the crops of a complete rotation, 1910–1913, from plots 1, 14, 26, and 36 (untreated) collectively, and from plot 4, dressed with potash, all of Tier III.

The following is a condensed summary of these crop analyses, with respect to the potash found:

Crop.	Untreated.		Dressed with potash.		Ratios of potash percentage in crop of plot 4 (plot 1=100).
	Weight of crop.	Potash.	Weight of crop.	Potash.	
	Pounds.	*Pounds.*	*Pounds.*	*Pounds.*	
Oats, 1904 (Tier IV)	2,320	26.17	2,360	38.86	146.3
Corn, 1910 (Tier III)	2,525	15.37	2,788	19.74	115.3
Oats, 1911 (Tier III)	832	9.81	1,040	18.01	147.0
Wheat, 1912 (Tier III)	2,669	12.16	2,172	11.97	120.6
Hay, 1913 (Tier III)	1,140	10.30	1,040	16.50	175.6
Total for 5 crops	9,486	73.81	9,400	105.08	143.7
Total for rotation, 1910–1913	7,166	47.64	7,040	66.22	141.9

In every one of these crops the percentage of potash was very markedly greater in that grown upon the plot dressed with potash, than in the corresponding crop grown upon the untreated plot 1, or upon its similars in treatment, plots 14, 26, and 34. The excesses on plot 4 for each unit weight of crop harvested varied from 15.7 to 75.6 per cent, and averaged 43.7 per cent for the five crops analyzed; 41.9 per cent for the four crops of 1910–1913, representing one complete rotation.

Since the potash fertilizer was applied just before the planting of the corn and wheat crops, it is perhaps not unexpected that these crops as harvested contain more potash than crops from lands receiving no fertilizer dressing. It is strikingly clear, however, that the larger excesses appear in the oats and hay crops, harvested 14 and 21 months, respectively, after the potash dressing had been applied. Furthermore, the excesses of potash in the plot 4 crops are possibly the more indicative of the ready availability of the potash in the soil of that plot from the fact that the crop yields were not increased by the potash fertilization.

The evidence from plant composition confirms, therefore, the indications obtained by the action of weak solvents upon the soil to the effect that of the potash in plot 4 much more exists in a state of availability to plants than of the potash in plot 1 despite the greater absolute quantity of the element present in the latter plot.

FATE OF THE FERTILIZER POTASH APPLIED

For an exact statistical allotment of the 1,800 pounds of potash applied to plot 4 from 1881 to 1916, inclusive, there would be required a number of data which the previously related studies have not furnished. We know that there is little loss or gain of the surface soil of this plot by erosive influences. The drainage is reasonably free, but there is no water table, for the drainage waters creep along the faces and through the crevices and seams of the limestone rocks that lie only a short distance beneath the surface. It has not been practicable, therefore, to collect and examine the drainage water lost from these two plots so as to determine the amounts of potash they severally lose through that channel. The general composition of drainage waters does not suggest that this loss can be large.

We have, moreover, no entirely satisfactory notion of the potash transfers from surface to subsoil on these plots. Little downward movement is indicated by the data at hand.

A crude approximation of the fate of the fertilizer potash is possible, nevertheless, from the data in hand if the losses by subsoil and drainage are treated as relatively small, and therefore negligible for the purpose of the computation.

The total yields (in pounds) to the acre, 1881–1916, for the respective rotation crops were, according to data supplied by Prof. C. F. Noll, of the Department of Agronomy of this Station:

Crop.	Plot 1, untreated.			Plot 4, potash-dressed.		
	Grain.	Straw.	Total.	Grain.	Straw.	Total.
Corn	18,144	14,784	32,928	21,072	19,000	40,072
Oats	8,002	11,709	19,811	9,236	13,215	22,451
Wheat	6,567	9,584	16,151	6,737	11,477	18,214
Hay			18,448			20,887
Total			87,338			101,624

If we estimate the potash removed by unit weights harvested of these several kinds of crops to have been, on the average, equal to the corresponding removals during the rotation 1910–1913, the total quantities thus taken from the two plots were:

Crop.	Percentages of crop.		Total weight (pounds).	
	Plot 1.	Plot 4.	Plot 1.	Plot 4.
Corn	0.609	0.708	200.5	283.7
Oats	1.179	1.732	233.4	388.9
Wheat	.456	.550	73.6	100.2
Hay	.904	1.587	166.8	331.3
	.....	.....	674.3	1,104.1
Excess removed from plot 4	.....	.....	.....	429.8

The excess removal by crops from plot 4 thus calculated is probably too great. The crop yields of plot 1 have been decreasing, but those also of plot 4 have been falling off at a similar rate. The potash stock of plot 1 has been drawn upon without replacement; that of plot 4 has been increasing. For, even at the rates apparent in 1910–1913, the average annual removals of potash in crops from the respective plots were: From plot 1, at the rate of 18.7 pounds to the acre; from plot 4, 30.7 pounds; whereas the average annual potash addition to plot 4 was 50 pounds to the acre. It is very possible, therefore, that the excess percentage of potash in a unit weight of harvested crop removed from plot 4 over that from plot 1 is now greater than in the earlier years of the experiment.

At the utmost, therefore, only 430 pounds of the 1,800 pounds of fertilizer potash applied to plot 4 has been removed in the crops harvested if, for this computation, we assume that the withdrawal from the original soil stocks were the same on each plot for each unit weight of a given crop harvested and that the excess removal on plot 4 is to be charged to the fertilizer potash. This leaves a balance of 1,370 pounds of fertilizer potash to be accounted for.

Upon the same basis of computation the crops of plot 4 used of the fertilizer applied only 12 pounds out of 50, or less than one-fourth. The smallness of this utilization is to be ascribed to the large natural supply of potash in this soil and the little crop increase the potash fertilizer here induces.

The actual weights of potash existing in the soils in different states of solubility remain to be considered. From data presented in an earlier section of this paper, the following facts appear as to the respective fine soil weights for the surface acre 7-inch layers and the total potash percentages:

	Plot 1.	Plot 4.
Weights of fine soil of acre 7 inches..pounds..	1,890,644	1,865,947
Total potash.....per cent..	3.821	3.543
Total potash.....pounds..	72,241	66,110

That is, the plot dressed with potash now contains, despite the fact that it received 50 pounds of potash a year, about 6,000 pounds less potash than the untreated plot. These differences in potash content of the two plots are doubtless due to their geological rather than their agricultural history. Their initial differences in potash content unsuit these two soils for any direct statistical comparison.

We may, however, assume that if plots 1 and 4 had had the same potash content at the outstart of the experiment and the same treatment since then the percentage proportion of this potash soluble under the conditions here studied would have been alike. To estimate the present differences in potash condition so as to exclude the influences of geological history and to represent only the effects of the differences in agricultural treatment, the respective solubility percentages for the potash in the contrasted plots are used as the quality factors and the present total potash in the plot 4 surface soil as the quantity factor. The present potash supply in the latter plot is slightly greater than it would have been if the potash additions had not been made. The residue of these additions is certainly not greater than 1,371 pounds, or approximately 2 per cent of the present potash stock. The influence of this amount upon these admittedly crude approximations has been deemed too slight for consideration in this computation. The potash weights thus computed to the acre are:

Treatment of potash.	Untreated soil.	Potash-dressed soil.	Excess in potash-dressed soil.
	Pounds.	*Pounds.*	*Pounds.*
Total potash	66, 110	66, 110	.....
Insoluble in hot, strong hydrochloric acid (1.115 sp. gr.) in 10 hours	59, 731	58, 512	.....
Soluble in 10 hours	6, 379	7, 598	1, 219
Soluble in *N*/5 hydrochloric acid, 5 hours at 40° C	247	562	315
Soluble in distilled water	78	149	71
Soluble in carbonated water	132	261	129
Soluble in *N*/3 ammonium-chlorid solution	163	349	186

It has already been observed that the natural stock of potash in the untreated soil is high. At the annual rate of removal in harvested crops for 1910–1913, the total supply would last 3,500 years, and that soluble in strong hot acid about 350 years. Even the quantities soluble upon a few hours' exposure of the soil to weak solvents would suffice for a number of years: The water soluble, 4 years; carbonated water soluble, 7 years; weak ammonium chlorid, 9 years; weak, warm acid, 13 years. Vigorous crops of normal quantity would, of course, remove more potash, but even for such growths the amounts readily soluble in the more or less carbonated soil moisture should amply suffice, judging from the observed solubility proportions. Experience on these lands has amply

demonstrated that only after years of normal crops have been removed is there any evidence of crop benefit by reason of potash applications.

The amounts of soluble potash in the soil that was dressed with potassic fertilizer are, no matter how weak the solvent used, relatively much greater than are found in the untreated soil. This fact, together with the composition of the crops grown on the respective lands, warrants the conclusion that the potash dressings remain, at least in part, readily available for some years after the application. The excess soluble in water corresponds to 1.4 times the annual addition; in carbonated water, 2.6 times; in weak solution of a neutral salt, 5.2 times; and in weak, warm acid, 6.3 times.

It is a curious coincidence that the excess of potash dissolved by hot, strong acid from the soil dressed with potash lacks very little of being equal to the amount of this element added, 1881–1916, less the excess quantity removed from plot 4 in the crops harvested (1,800–430 = 1,370 pounds). That this closeness of agreement is merely a coincidence must be evident from what has already been remarked concerning the large effects upon the strong acid extraction of soil potash that are observed when the time or temperature conditions are slightly changed. Frear and White (*4, p. 187*) have reported, from analyses of the sod lands adjacent to and intersecting the tiers of plots under the general series of experiments, that while the Association method of acid treatment removes somewhat over 8 per cent of the potash of these sod-land surface layers, Hilgard's method, which requires a 5-day instead of a 10-hour treatment, increases to 22.7 per cent the proportions of the total potash removed.

Finally, it should be noted that, although the crops harvested from plot 4 removed only two-ninths of the fertilizer potash applied, only three-eighteenths of the quantity applied remains in such condition that it can be readily dissolved by warm, weak acid. These quantities leave eleven-eighteenths of the applied amounts to be otherwise accounted for. The figures for potash soluble in strong acid do not indicate that much of this potash, except what the harvested crops took away, has been removed from the surface soil. Most of it remains there in rather difficultly soluble condition. In other words, the residual potash tends to assume, in large part, a condition of relatively slow availability. Concerning the time relations of this change, little is known. Gilbert (*5*) remarked a similar change in the solubility of the residues of fertilizer potash in the loam soil at Rothamsted.

SUMMARY

A comparison, as to the condition of the potash in a Hagerstown silty loam soil which has in the past 36 years received, in 18 equal biennial dressings, 1,800 pounds of fertilizer potash, with that in a neighboring

portion of the same soil which has, during the same period, received no fertilizer addition of any kind, but which has been tilled and cropped the same, gives the following results:

(1) The proportion of the potash dissolved by strong, hot acid is somewhat greater where the potash dressings have been used. In weak solvents (distilled water, carbonated water, weak solution of ammonium chlorid, and *N*/5 hydrochloric acid) twice as much potash is dissolved in a short time at moderate temperatures from the fertilized soil as these solvents take, under the same solution conditions, from the unfertilized soil.

(2) Of the weak solvents named, the *N*/5 acid dissolves the most potash. The quantities of this element dissolved by the other weak solvents, differ, of course, with the solvent; but each forms in each soil the same percentage proportion of the amount dissolved by the *N*/5 acid—that is, the solvent effects are parallel.

(3) The potash dissolved upon a second extraction with ammonium-chlorid solution is very much less than is dissolved by the first extraction with this solvent, and the quantities are practically the same for each soil; whereas that removed by the first extraction from the soil dressed with potash is twice as great as the amount taken from the unfertilized soil.

(4) The clays and nonclays of these soils, after separation by sedimentation in water, show the following characteristics as to potash content and solubility: The clays contain less potash than the nonclays. The clay of the potash soil is richer in potash than that of the unfertilized soil. The same is true, but in less degree, of its nonclay fraction. Unit weights of the clays give up much more potash to ammonium-chlorid solution than unit weights of the nonclays. Both fractions of the potash-dressed soil exceed those of the unfertilized soil in this respect, but the clays much more than the nonclays. Unit surface areas of the clay particles, as conventionally calculated, give up much less potash to the solution than equal surface areas of the nonclays. This may, however, be due to a reduction of free-clay surface to less than the conventional area by cementing of the particles in drying, or because of the flocculating influence of the saline solvent used.

(5) The soil is naturally rich in potash, and potash dressings cause little or no crop increase. These dressings are followed, however, by an increase in the amounts of potash taken up by the crops. Five crops examined all show this increase. On the average for a rotation, the crops harvested from the land dressed with potash carry off in a given weight of harvest, 40 per cent more potash than a like harvest weight from the unfertilized land contains—that is, both chemical solvent and plant agree in indicating a higher availability for at least part of the potash in the potash-dressed soil. Moreover, the crops grown the

second year after the application show a greater potash excess than those to which the potash fertilizer is directly applied.

(6) On crediting the fertilizer potash with the excess only of the potash in the crops from the fertilized soil, the crops have used not more than one-fourth of the potash dressings applied, leaving a residue of 1,300 to 1,400 pounds of fertilizer potash to be otherwise accounted for. The higher solubility in weak solvents of the potash in the fertilized soil accounts for enough of this residue to correspond to crop requirements through a few years. The larger amount of potash dissolved from the fertilized soil by strong, hot acid accounts for practically the entire residue, but the close correspondence in potash quantities here observed is doubtless an accident due to the particular conditions of temperature and duration of solvent action maintained.

(7) Taken as a whole, the conclusion is warranted that much of the potash applied as fertilizer remains in the surface soil in a state highly available to crops; that most of it remains there in a condition of lower availability, and that the losses by drainage have probably not been great.

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HARDENING PROCESS IN PLANTS AND DEVELOPMENTS FROM FROST INJURY [1]

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INTRODUCTION

Hardening plants to resist frost injury is a well-established practice. The physiological basis for this practice and the mechanism of frost injury have formed an interesting chapter in plant physiology, for it seems that the more commonly understood physical phenomena play an important rôle in determining the resistance of plants to frost. In the resistance of a plant to freezing the relative importance of such factors as undercooling of the tissue, the freezing point of the cell sap, and the precipitation of proteins is much disputed, owing probably to the fact that different plants have been investigated by the various authors.

In the investigation reported in this paper experiments were made to determine the physiological changes found to occur under this treatment in such plants as cabbage (*Brassica oleracea capitata*), and tomatoes (*Lycopersicon esculentum*).

The method of hardening commonly used is to expose the succulent plants in coldframes for a week or more to temperatures somewhat above the freezing point.

REVIEW OF LITERATURE

The mechanics of the process of ice formation within tissues have been investigated by Duhamel and Buffon (*9*).[2] They ascribed frost injury to rupture of the cells by growing ice crystals. Göppert (*12*) and Sachs (*42*, *43*), however, found that ice formation takes place mostly in the intercellular spaces, and hence rupture of the cells does not cause injury from freezing. Müller (*37*, *39*) reported that ice formation within the cell takes place only on rapid cooling and that ice formation within the tissue was necessary to produce a true frost injury. Wiegand (*48*, *49*) observed microscopically the point of first formation of ice crystals and their increase in size in the intercellular spaces.

[1] In the latter part of 1915 the work here presented was undertaken at the suggestion of Dr. R. H True, Physiologist in Charge of Plant Physiological Investigations, to whom the writer owes much for advice and direction. The writer is also indebted to Dr. William Crocker, Dr. S. H. Eckerson, Dr. F. C. Koch, and other members of the faculty of the Hull Biological Laboratories of the University of Chicago for instruction and for the use of equipment at that institution.

[2] Reference is made by number (italic) to "Literature cited," p. 108–111.

Undercooling was found by Mez (*33*) to be a factor of great importance in frost injury. Where ice formation occurs at once, the lower tissues of trees are protected by the poorly conducting layers of frozen tissue on the outside. Voigtländer (*47*) showed that, owing to the continual movement of plants in the wind, but little undercooling of the cell sap occurs in nature.

A mechanical effect of ice formation upon the plasma membrane has been ascribed by Maximow (*30*, *31*, *32*) as a cause contributing to the frost injury of cells. He stated that the osmotic properties of the plasma membrane are changed by freezing, being supported in this opinion by Chandler (*5*).

Molisch (*36*) and Müller (*37*, *39*) ascribed frost injury to the withdrawal of water from the plasma membrane, and with this view Maximow partly agrees. The former authors did not follow the effect of desiccation farther than to state that the injury is due to the withdrawal of water during freezing. The processes of freezing, desiccation, and plasmolysis were found to be analagous in their effects upon the cell by Matruchot and Molliard (*28*, *29*). The analogy of these processes is further indicated by the work of Greely (*14*).

Gorke (*13*) advanced the idea that frost injury is due to the precipitation of proteins through salting out. He considered the concentration of the salts of the cell sap on freezing to be sufficient to precipitate irreversibly the proteins in solution in the cell sap and to cause similar changes in the protoplasmic gels. He considered the effect of the increased concentration of acid salts to be small, however, and insufficient to account for the precipitation. He was supported in this statement by later workers, including Schaffnit (*44*) and Lidforss (*23*). Voigtländer (*47*) doubted that the salting out of the proteins accounts for frost injury. Chandler (*5*) maintained that protein precipitation does not occur to any significant degree. He found that plants increase in hardiness when given nutrient salts in abundance, while, in accordance with Gorke's (*13*) theory, these plants should be more easily injured, owing to the increased salt content of their cell sap. It therefore appears that some factor other than salting out is necessary to account for the injury.

The accumulation of sugars and consequent increased depression of the freezing point of the cell sap was observed by Müller (*38*) in plants exposed to low temperatures. The effect of this increased content of sugars was followed by Lidforss (*23*), Schaffnit (*44*), Kovchoff (*21*) and Bartetzko (*1*). All of these authors agree in ascribing to sugars an important rôle in the prevention of protein precipitation owing to its protective effect for colloids.

The lesser injury from freezing shown by hardened plants was ascribed by Gorke (*13*), Schaffnit (*44*), and Bartetzko (*1*) to changes in the proteins. Schaffnit thought these changes consisted in a cleavage of the "high

molecular" forms of the proteins to simple forms, which are not so easily precipitated by the salting-out process.

In the plants which have been tested in this study all of these factors seem to play a part. In addition to these, there appears another important factor, which for the most part has been disregarded, the change in the actual acidity or hydrogen-ion concentration of the plant juice on freezing. It seems that this factor supplies the deficiencies of the other factors in explaining frost injury.

FIRST INDICATIONS OF FROST INJURY AND THEIR DEVELOPMENT IN CABBAGE AND TOMATO

The first indication of frost injury in the herbaceous plants which have been observed is in the appearance of injected areas. Where the exposure to low temperature has been of short duration, these areas appear as isolated dots over the surface of the leaf, as is well shown by cabbage and tomato (Pl. 7); and occasionally along the stem also, observed on sunflower (*Helianthus annuus*). The injected spots when observed by transmitted light are more transparent than the remainder of the leaf. The transparency is due to the displacement of air, which is ordinarily present within the intercellular spaces of the spongy parenchyma, by water which has been withdrawn from the cells during the process of freezing. It was shown by Sachs (*42*, *43*) that in the process of freezing, water passes out from the protoplast and freezes in the intercellular spaces. On thawing, this water is then left in the intercellular spaces until such time as it is evaporated or reabsorbed by the cells which have been plasmolyzed by freezing. Under certain conditions it requires considerable time for the protoplast to return to its former position against the cell wall and to regain its turgidity.

The tomato leaf does not survive ice formation in the tissue; consequently the injected spots appear as brown areas after a few hours. Sections of these spots made immediately after freezing show a collapsed condition of the palisade cells (Pl. 8, A). The collapse of the palisade causes depressions in the leaf surface, and these areas dry up after a few days. Death of the injured cells produces the spotted appearance shown in the leaf at the left of Plate 7, B. The cells around these areas, although exposed to the same temperature, show no injury because there was no ice formation. The effect of short exposures to low temperature appears to be nil, the injury being an accompaniment of ice formation. Voigtländer (*47*) has previously shown that there must be ice formation in the tissues to produce frost injury.

MECHANISM OF FREEZING

The undercooling of the cell solution is a factor of great importance in the resistance of cabbage to freezing. Those plants which have the most bloom on the leaf surface are most resistant to the formation of

ice within the tissue. Cabbages which are well covered with wax show no indications of freezing after several hours' exposure to a temperature 5 degrees below that at which ordinary plants show ice formation.

When one considers the physical mechanism of freezing in the leaf, the cause of the resistance is found to be this coating of wax. In the natural state moisture is usually present on the surface of leaves, even though in minute quantities. This may be deposited by condensation from the surrounding atmosphere. In cooling down to only slightly below zero this water freezes, for the plant is seldom motionless enough to allow much undercooling, as shown by Voigtländer (47). In plants which have but little wax greater amounts of moisture stick to the leaves, while those covered with wax are not wet. This can be observed if such leaves are immersed in water. Those with a thick coating of wax have a bright silvery sheen, owing to the lack of wetting of the leaf surface. If the wax is rubbed off, the surface then becomes wet and loses its ability to form a mirror surface.

Water which freezes on the leaf surface serves to inoculate the undercooled solution within the leaf; in fact, the injected spots observed are caused by this inoculation. When once begun, the freezing process is transmitted rapidly through the undercooled leaf tissue, and the frozen spot enlarges until the whole leaf may be frozen. That water on the surface may cause the inoculation is easily shown by placing a drop of water on the leaf and exposing it to $-3°$ C., when the area beneath the drop will be found to freeze first and show injection. Inoculation probably takes place through stomata or through cracks in the wax and will take place less frequently if the wax is thick.

Bigelow and Rykenboer (2) have recently shown that a very great undercooling can occur in capillary tubes. Stomata and cracks in the waxy covering of the leaf are of small enough dimensions to allow considerable undercooling without ice formation taking place through them to inoculate the tissue beneath. It would appear consequently that where inoculation occurs the openings are largest.

Freezing in spots is of common occurrence in plants. It has been observed to occur on cabbages when they are exposed to frost in the open. The injected areas have been produced on various greenhouse plants, including begonia, salvia, geranium, coleus, bryophyllum, lettuce, sunflower, hydrangea, and *Aucuba japonica*.

Unhardened cabbage leaves survive ice formation within the tissue when the injected area does not cover too great a part of the surface. Only slight plasmolysis can be observed in sections of the injected spots in this case. On fixing with acid alcohol (Carnoy's solution) cabbage leaves which have been frozen in spots, large masses of spherocrystals were found, quite sharply limited to the injected areas and often not to be found in the rest of the tissue. These crystals (Pl. 8, B) appear to be

calcium malophosphate. Evidence of the identity of this substance is given by the spherocrystalline form (*46*); its reaction with ammonium molybdate and magnesia mixture, showing the presence of phosphates; replacement of the spherocrystals by gypsum crystals on treatment with dilute sulphuric acid; carbonization on treatment with concentrated sulphuric acid; slow solubility in water; solubility in saturated solutions of dicalcium and tricalcium phosphate and insolubility in saturated monocalcium phosphate. Crystals of maleic acid were obtained by microsublimation from cabbage leaf. After fixing in Carnoy's solution these crystal patches are to be found in great abundance in the older leaves, which are not frozen as easily as the leaves up to an inch in length. The crystals are more abundant in hardened than in nonhardened cabbage leaves of the same size. Precipitation of the malophosphate in the injected areas seems to be caused by greater concentration there, owing to some effect of the freezing. Since Carnoy's solution kills the tissues very rapidly, it is not probable that a diffusion from the surrounding tissue into these spots would occur to any great extent.

GROWTH DEVELOPMENT IN THE INJECTED AREAS

On standing at room temperature for a few minutes the injected areas of cabbage often disappear, and no trace of them can be seen for two or three days after freezing. At about the third day the spots again become evident as slightly raised areas sharply defined. The raised portions are a little lighter in color than the rest of the leaf. A decrease in the number of chloroplasts in these areas gives the leaf a mottled appearance. Similar conditions have been observed by Ritzema Bos (*41*) on other plants of this genus (*Brassica napus*) as a result of frost injury. The young intumescences grow very rapidly for 10 days or more and may reach a relatively enormous size, showing in section a thickness many times that of the normal leaf. They are often of a circular shape, but they may have any shape, corresponding to the coalescence of the injected areas as they increase in size (Pl. 9). When a large portion of the tissue is injected, it is difficult to keep the entire leaf from dying, but death may be prevented by placing the plant in a saturated atmosphere. The entire leaf may be a mass of intumescences so that it is rolled and thickened in all manner of shapes. The swellings occur along the veins of the leaf more abundantly than over the remainder of the leaf surface (Pl. 9, B), although the portions about the veins seem no more liable to injection than other parts.

In section the tumors when about 4 days old are seen to consist of enormous cells with large nuclei (Pl. 8, C). These large cells are often bi- or tri-nucleate, a condition commonly observed in pathological conditions. The walls of these cells are quite thin, and large vacuoles appear in the protoplast. The peculiar large cells recall the pathological

condition of cells recently found by Samuels [1] to accompany the process of precipitation of raphides of calcium oxalate.

Quite often in older intumescences there seems to be a return to the meristematic condition in certain cells, so that a cambium-like layer is formed which pushes out conical-shaped rows of cells to form the tumor (Pl. 10, A). After two weeks' growth the tumors begin to die back, and infection may occur (Pl. 10, B). Before this time no bacteria have been observed in the formation of the tumor.

Growth is sharply confined to the area injected and does not spread to the other cells. Such limitation would hardly be the case if bacteria were concerned in the growth stimulus. The growth is not due to any condition peculiar to the cabbages used, for identical growths have been obtained during different years. Similar intumescences have been produced from frost-injured spots on the leaves of *Bryophyllum calicynum*.

Small growths have also been observed to be produced from injected areas on the leaves of lettuce and salvia. The cells of injected areas of hardened cabbage leaves are not stimulated to growth by moderate freezing. Since plasmolysis of the cells occurs in this case, as well as in the cells of nonhardened plants, it appears that plasmolysis alone is not the cause of the growth stimulus.

Young intumescences contain large quantities of dextrose, much more than the ordinary leaf cells. Levulose is present in only small quantities, if at all. When heated with Fehling's solution, an abundant precipitate of cuprous oxid is formed in the tumors, giving them a reddish color. Starch and tannins are absent from the hypertrophied areas.

PEROXIDASE CHANGES IN THE INJECTED AREAS OF CABBAGE LEAVES

A lot of cabbage leaves were spotted and placed in the greenhouse to allow the development of tumors. From time to time samples of the spotted leaves were tested for peroxidase. Tetramethylparaphenylenediamin in 60 per cent alcohol was used as the reagent for oxidase, and 10 per cent of commercial hydrogen peroxid was added to this to demonstrate the peroxidases. In these reagents the activity of the solutions was tested on sections of potatoes on which known reactions were given. Little or no reaction for oxidase was obtained in cabbage leaves within the time in which potato sections gave a good reaction. This was supported by testing the leaf juices in the Bunzell (*4*) apparatus, which showed comparatively little oxidase activity with tyrosin, pyrogallol, hydroquinone, and pyrocatechin. It was found that with tetramethylparaphenylenediamin a good test for peroxidase was given along the veins of the cabbage leaf. In leaves tested immediately after spotting no more peroxidase reaction was given in the injected than in other areas; nor could any greater amount of peroxidase be demonstrated in

[1] Paper read before the American Association for the Advancement of Science, New York, 1916.

the spots until the growth of the intumescences began. At about the second or third day after freezing, the spotted areas showed more or less irregularly an increase in the peroxidase reaction. In young tumors which were growing rapidly there was a marked increase in the peroxidase reaction of the tumors over the rest of the leaf, with the exception of the veins. Plate A gives an idea of the relative peroxidase reaction shown by the purple color in the tumors as well as the relative color of tumor and normal areas of the leaf before applying the reaction. In all cases leaves which were plunged into boiling water for two minutes gave negative tests for peroxidase.

Woods (*50*) found greater amounts of peroxidase in the spotted areas of tobacco leaves infected with mosaic than in the normal areas of the leaf. He attributed the lighter color to the oxidation of the chlorophyll by the abnormally increased peroxidase. He submitted evidence to show that chlorophyll is destroyed by peroxidases *in vitro*. It is possible that there is a similar connection in the case of tumored cabbage leaves and that this accounts for the lighter color of the intumescences.

These abnormal growths were first obtained in the winter of 1915 in great abundance and at will. The only conditions necessary are to have the temperature to which the cabbages are exposed so regulated that the freezing can be stopped before the leaf is killed by being frozen throughout. The temperature required for cabbages is about $-3°$C., with an exposure of about 30 minutes. In June, 1916, Smith (*45*) began some experiments on the production of abnormal growths by injecting certain simple chemical substances or exposing plants to the vapor of these substances. This interesting work has already been reported. The chief point in his report to be noticed here is that a number of these substances, ammonia, acetic acid, amins, etc., are either acid or alkaline in reaction, and some strongly so, a point which will be discussed later. Among other plants tested, cauliflower plants (*Brassica oleracea botrytis*) were exposed to ammonia vapor. The intumescences which resulted might well be taken for those shown in the illustrations in this paper, so similar are they. The differences seem to be that on exposure to ammonia a smaller area is affected, such as that about a stomatal opening. In Plate 63 of Smith's report (*45*) the growths appear along the veins in abundance. A similar stimulus can be given by freezing, a purely physical process at the start, as well as by chemical treatment, indicating a similar basic cause in the two processes.

Intumescences of this kind are quite commonly found on cabbage, resulting from aphis punctures and from breaking the leaf surface; injections of water which loosen the epidermal layer produce them; in fact, it is only necessary to puncture the epidermis to cause their development. Hence, it seems that the growth response is a common phenomenon in cabbage and that it may be caused by a condition common either to

exposure to acid or alkaline vapors, to freezing, or to drying by excessive transpiration from exposed areas. The factor active here seems to the writer to be a change in the reaction of the plant juice, accompanied also by an increase in the concentration of the salts of the cell sap. That a change of acidity might occur from exposure to ammonia or acetic-acid vapor is not difficult to see. The change in reaction of the plant juice on concentration by transpiration is shown by the following experiment:

Fresh cabbage leaves were taken and divided into two lots. The first half was kept in a cool, moist atmosphere; the other half was allowed to wilt in a dry room until turgidity had been lost. Then both samples were ground in a meat chopper, and the juice was expressed. The acidity of the leaf juice was increased by the wilting from 2.05×10^{-6} to 5.37×10^{-6} H^+. Evidence of the change of reaction on freezing will be given later.

By placing plants which have been injected in spots into tap water for from 5 to 12 hours the formation of tumors can be stopped. This inhibition was accomplished by inverting a potted cabbage over a beaker of tap water so that all or part of the leaves were immersed. The coating of wax on the leaves prevents their wetting by the water and holds a layer of air about the leaf. This treatment effectually stops transpiration, although it may also produce abnormal conditions, such as a poor oxygen supply. When treated in this manner, the injected areas fail to develop into tumors, and this hindrance is confined to those spots which are immersed. It is interesting in this regard to note the old observation of Sachs (*42*, *43*) that slow thawing, as he regarded it, decreased frost injury. His method was to place the frozen tissue into water and allow it to thaw out slowly. Müller (*37*, *39*) showed that this process in fact caused a very rapid thawing, owing to the rapid thermal exchange. Hence, the lessened injury must rest on some basis other than slow thawing of the tissue. Greenhouse men have generally observed that frost injury is much less if the frosted plants are at once sprinkled thoroughly.

PLASMOLYTIC BEHAVIOR OF TUMOR CELLS

The cells of young cabbage tumors are plasmolyzed at lower concentrations and in shorter time than the mesophyll cells in adjoining normal tissue. Sections were made from fresh tumors, thin enough to be observed by the low power of the microscope and still thick enough to leave several uninjured cells. It was found to give greatest regularity if the tumor cells were compared with the normal mesophyll cells from the same section for their plasmolytic behavior. Observations on the plasmolytic limits of such tissues are very difficult and are mainly comparative rather than to be taken as a measure of actual osmotic pressure or permeability.

Table I shows the plasmolytic limit concentrations after 30 minutes' exposure.

TABLE I.—*Plasmolytic limit concentrations for normal and tumor cells*

Kind of cell.	Freezing points of solutions.			
	Glycerin.	Sucrose.	Potassium nitrate.	Calcium nitrate.
	°C.	°C.	°C.	°C.
Normal mesophyll	−1.943	−3.290	−1.733	−1.462
Tumor cells	−1.132	−1.509	−1.461	−.952

These values are for young tumors about 5 days old and in active growth. The age of the tumor has much to do with its plasmolytic behavior. Very old tumor cells which have stopped growth show no difference in plasmolytic behavior from normal mesophyll cells of the same leaf. The writer is inclined to regard the difference in the plasmolytic behavior as being due, not to a difference in the osmotic concentration of the cell sap, but rather to differences in permeability, especially to water, and probably also to the substance used for plasmolysis. Smith (*45, p. 184*) says:

> . . . it would seem . . . that in local osmotic action (possibly in some stages chemical action also) of various substances . . . we have . . . the explanation of tumor growth. . . .

This statement evidently implies a changed permeability as well as pure osmotic effect. The writer has determined the freezing points of juices expressed from cultures of tumors caused by *Bacillus tumefaciens* grown on daisy from Smith's cultures, and has found a lower freezing point for tumor tissue than for either stem or leaf tissue. Evidence offered by the greater freezing-point depression of saps of parasites than of saps from the host (*17*) seem to indicate that such an excess depression is a common condition for parasitic tissues (*24*). The accumulation of such large quantities of sugar in the tumors of cabbage caused by freezing leads the author to believe that there is some change in the cells which allows them to obtain it from their neighbors in the surrounding areas. The quantities of starch present in the cells before freezing are not sufficient to account for this much sugar, for the young leaves in which tumors develop rapidly contain very little starch. The decrease in chlorophyll would hardly be in accord with the synthesis of the sugar within the tumor cells.

It is probable that in the frozen cells of the injected areas there occur changes in the state of the protoplasm constitutents so that more sugar is adsorbed there, or so that the sugar is held there in some sort of chemical combination. Osterhout (*40*) suggests that differences in

permeability alone do not effect the accumulation of substances within cells.

So far the writer has not obtained the freezing point of juice expressed from cabbage tumors caused by frost injury. However, some evidence of the relative freezing points of the tumor cells and leaf cells can be gained from the following experiment: Cabbage plants showing an abundance of young tumors were placed at a constant temperature just sufficiently low to freeze the tissue. It was observed that on freezing the leaves were frozen in spots over the areas not covered by tumors usually before the tumor cells were frozen. One would expect the tumors to freeze much more quickly than the normal tissue if the relative osmotic concentrations were those shown by plasmolysis. The failure of the tumors to freeze can not be attributed to greater undercooling in them, for the cells have thin walls and are not covered by wax.

PHYSIOLOGICAL CHANGE IN CABBAGES DURING THE HARDENING PROCESS

The physical changes in cabbage which have been observed during the hardening process are a slowing of the growth rate so that the plants are smaller than those of the same age grown at a warm temperature and consequently are more mature; and an increase in the amount of bloom on the leaves. The condition of hardiness can well be judged by the stiff, springy condition of the leaves. Hardened leaves are 20 per cent thicker than nonhardened leaves of the same age.

By exposure to temperatures a few degrees above the freezing point for a week or so the Early Jersey Wakefield cabbage acquires the ability to be frozen stiff without injury, or with only slight injury (Pl. 11, A). Plants frozen directly after being taken from the greenhouse are either injured in spots or killed throughout, according to the extent of the frozen area and the temperature. Tomatoes under similar hardening treatment can be made to withstand somewhat lower temperatures without freezing; but once the hardened plants become frozen they are killed (Pl. 11, B). Tomatoes are killed or injured on long exposure to a temperature of 5° C. This injuring is not a true freezing to death, according to Müller (*37, 39*), for no ice formation takes place in the tissue. Molisch (*36*) explains it on the basis of accumulation of toxic substances through poor oxidation. Tomatoes which can not survive ice formation within the tissue can hardly be said to show a true hardiness to frost. The term "hardiness" should be applied to the ability of a plant to survive ice formation within its tissues, as shown by cabbage.

A condition of greater resistance to freezing can be produced in cabbages by watering them with solutions which check growth. Plants watered with nutrient solutions which produce rapid growth, such as *N/10* potassium nitrate, *N/10* calcium nitrate, or Knop's 2 per cent, are

much more easily injured than those watered with *N/10* sodium chlorid or *N/10*, sodium bicarbonate. The latter solutions, like exposure to low temperature, retard growth. Cultures watered with all the foregoing *N/10* solutions showed a lower freezing point than controls grown in poor clay soil. An increase in the cell-sap concentration and lowering of the freezing point can also be produced by growing plants in dry soil. The increased resistance of cabbage watered with salt solutions indicates that it is not the increase of concentration of just the salts of the cell sap on freezing that causes injury.

METHOD OF TESTING HARDINESS

An electrically-controlled constant-temperature room which can be maintained at any desired temperature within a few tenths of a degree was used in determining the hardiness of the plants under test. This compartment was cooled by the direct expansion of ammonia. The temperature was kept the same throughout by keeping the air stirred vigorously with an electric fan. Plants were exposed to a temperature just sufficient to freeze them, and they were then observed at intervals for the appearance of injury. Comparative tests were run together and for the same length of time.

TIME AND TEMPERATURE FACTORS IN ACQUIRING OR LOSING HARDINESS

The time necessary for plants to become hardened was determined by placing cabbage in dark chambers at constant temperatures of 3°C., and 5°, using 18° and 25° as controls. Exposure for 24 hours to 3° was found to produce a slightly increased hardiness as judged by the extent of the injury. After 5 days' exposure to 3° the cabbages were not injured by 30 minutes' exposure to −3°, although frozen stiff. The control plants were killed throughout. On placing such hardened plants in the greenhouse at room temperature and at a constant temperature of 18° in the dark, it was found that the hardiness was lost in the greenhouse in about the time taken to acquire it, while the hardiness lasted a few days longer at 18°. By alternating cabbage plants between 3° and 25° it was shown that the hardening process is an accommodation brought about not by changes of temperature such as occur in the natural hardening of plants, but by low temperature. From the above statements it is seen that plants acquire and lose hardiness rather rapidly. Under natural conditions the hardiness acquired in one night of low temperature may be lost during the succeeding warm day, and there is accumulative effect only when the average temperature is low.

The maturity of the tissue is a factor of great importance in frost resistance. Young leaves of cabbage are more easily injured than old leaves. During hardening these young leaves become resistant, indi-

cating that they rapidly pass through some sort of maturation process. The more common injury to the cells about the veins indicates that there is a physiological difference between the vascular tissue and the other cells of the leaf. This physiological difference is suggested also by Mangham (*27*) in a recent article.

FREEZING POINT AND EXPRESSION OF CABBAGE JUICES

A large number of determinations were made of the freezing points of juice expressed by different methods from hardened and nonhardened plants. As Dixon and Atkins (*8*) have shown, the method of treatment before expression has a considerable influence on the depression values. However, there is about the same difference between the freezing points of juices expressed from hardened and nonhardened cabbages regardless of the method of treatment before expression. Tests were made after freezing in liquid air, after freezing with solid carbon dioxid, after freezing at −5° C., and without freezing. One of the most convenient methods is to freeze the tissue with carbon dioxid until brittle and then to grind it in a mortar to a fine powder. The material can then be transferred to the press and allowed to thaw out. This method is more available than the liquid-air method and is less expensive. The sap was expressed either in a large hand press which left a marc practically dry or in a hydraulic press under a pressure of 10 to 30 tons on a 2¼-inch ram. Table II gives the comparative values.

TABLE II.—*Depression values for cabbage juice*

Condition.	Not frozen.	Method of freezing before expression.		
		−5 °C.	Carbon dioxid.	Liquid air.
	°C.	°C.	°C.	°C.
Hardened	−0.985	−1.160	−1.630	−1.822
Nonhardened	−0.910	−1.122	−1.530	−1.668
Excess depression of hardened over nonhardened	−0.076	−0.036	−0.080	−0.154

Average excess depression of hardened over nonhardened, 0.085°C.

The smallest difference, 0.036° C., shown after freezing at −5° may be due to a greater injury to the nonhardened leaves and corresponding increase in concentration of the expressed juice. The increase of the freezing-point depression on treatment with solid carbon dioxid and liquid air over that given by freezing at −5° may be due to changes in the cell membranes which make them more permeable to osmotically active substances. The process of freezing, especially to this degree, is no innocent procedure and may result in changes which produce protein precipitation. Consequently, juices expressed in this manner do not represent the true concentration of the cell sap for all its constituents.

It is quite clear that the difference of about 0.1 degree in the freezing point of the cell sap from hardened and nonhardened cabbages is insufficient to account for the resistance to low temperature shown by hardened plants, for hardened plants are not injured by being frozen at a temperature 3 degrees below the killing temperature for nonhardened plants (*6*). Since ice formation takes place in this case, the freezing point of the cell sap must be exceeded.

CHEMICAL CHANGES DURING HARDENING

CARBOHYDRATES.—In studying the carbohydrate changes during the hardening process cabbage plants from the same lot were placed part in the greenhouse and part in a coldframe. The plants were allowed to harden in the coldframe until they were not injured by being frozen stiff at −3° C. Three samples of leaves, 25 gm. each, were taken from each lot for starch and sugar determinations. The percentage values are shown in Table III.

TABLE III.—*Analyses of carbohydrates in cabbage* [a]

Sample No.	Reducing sugars as glucose.	Dissaccharids as sucrose.	Polysaccharids as starch.
Hardened cabbages:	*Per cent.*	*Per cent.*	*Per cent.*
A1	3.13	1.91	2.69
A2	3.03	2.05	2.75
A3	2.56	2.33	2.45
Average	2.91	2.10	2.63
Nonhardened cabbages:			
B1	1.66	.21	3.36
B2	1.54	.19	3.39
B3	1.48	.18	3.41
Average	1.56	.19	3.39

[a] The writer is indebted to Mr. J. W. Kelly, of the Office of Drug-Plant, Poisonous Plant, Physiological and Fermentation Investigations, for the carbohydrate determinations.

In the average of these values it is seen that the equilibrium between starch, glucose, and sucrose in the nonhardened plants in which polysaccharids predominate is displaced in hardened cabbages in the direction of the mono- and di-saccharids. Lidforss (*23*) found this to be a common transformation in plants during the cold season. Hasselbring and Hawkins (*18*) have found a similar condition to occur in sweet potatoes kept at low temperatures. They submit evidence to show that the increase in sucrose takes place only after glucose formation from the starch. It is difficult to maintain cabbage plants without photosynthesis for a sufficient time to observe if that is the case here.

The writer has been unable to find any considerable amount of levulose in cabbage leaves by microchemical means.

The cleavage of polysaccharids yields osmotically active substances which account for the lower freezing point of juices expressed from hardened plants. As Schaffnit (*44*) has shown, the sugars produced may have a considerable influence on frost resistance by allowing the solution to be undercooled to a greater degree or by preventing the precipitation of proteins.

The carbohydrate changes were determined also in plants kept at constant temperatures in the dark to determine the relation of carbohydrate change to the time of acquiring hardiness (Table IV).

TABLE IV.—*Analyses of carbohydrates in cabbage plants kept at constant temperatures in the dark*

Temperature.	Duration.	Starch.	Reducing sugars.	Sucrose.	Freezing point of sap.	Condition of hardiness.
°C.	*Days.*	*Per cent.*	*Per cent.*	*Per cent.*	° C.	
Greenhouse		1.72	0.70	0.076	−0.816	At −2.5° C. completely killed in 60 minutes.
18	5	1.72	1.00	.00	− .675	At −2.5° C. two-thirds killed in 60 minutes.
5	5	1.64	1.09	.066	− .725	At −2.5° C. not injured in 60 minutes. Frozen stiff.
18	10	1.19	.48	.16		At −4.5° C. all killed in 60 minutes.
5	10	1.33	.98	.015		At −4.5° C. one-third killed in 60 minutes. Frozen stiff.
Greenhouse		1.37	1.17	.06	− .780	At −3° C. all killed in 30 minutes.
25	6	1.33	.76	.15	− .746	At −3° C. all killed in 30 minutes.
3	6	1.19	.72	.19	− .797	At −3° C. not injured in 30 minutes. Frozen stiff.
25	10	1.00	.16	.05		
3	10	1.30	.80	.15		

From Tables III and IV it is seen that hardiness is acquired before any great change occurs in the carbohydrate equilibrium. A utilization of both starch and the reducing sugars in cultures kept at higher temperatures is noticeable, as well as the lack of any great change at the lower temperatures.

Analyses of hardened and nonhardened cabbages were also made by a modification of Koch's (*20*) method. This method involves a separation

into a lipoid fraction (F_1), a water-soluble fraction (F_2), and a fraction insoluble in alcohol and water (F_3). Samples of 100 gm., green weight, were collected from hardened (H_1, H_2) and nonhardened (NH_1, NH_2) cabbages and preserved in 85 per cent alcohol with the addition of 0.5 gm. of calcium carbonate. The weights given in grams in Table V express percentages on the basis of green weight.

TABLE V.—*Complete analyses of hardened and nonhardened cabbages*

Constituent.	H_1	H_2	NH_1	NH_2
Total solids	10.1844	10.5604	9.035	9.2945
Moisture	89.8186	89.4396	90.965	90.965
Total phosphorus	.0669	.0664	.0535	.0537
Total nitrogen	.3015	.3024	.2986	.2915
Total lipoid phosphorus	.0124	.0121	.0089	.0093
Total lipoid nitrogen	.0112	.0129	.0146	.0123
Organic solids F_2	1.5037	1.6137	1.0859	1.2750
Phosphorus F_2	.0023	.....	.0029	.0041
Nitrogen F_2	.....	.0445	.0459	.0464
Ammonia F_2	.0010	.....	.0007	.....
Total Van Slyke nitrogen	.0168	.0162	.0084	.0068
Organic solids	5.8603	6.0869	4.8756	5.0593
Phosphorus F_3	.0522	.0520	.0417	.0403
Nitrogen F_3	.....	.2458	.2381	.2328
Water-soluble phosphorus F_3	.....	.0314	.0265	.....

The percentage of moisture in hardened cabbages is decreased slightly with a corresponding increase in the organic solids of the third fraction (F_3). It is difficult to obtain the same degree of humidity under such different conditions as used for hardening, and this may well account for a difference of 1 per cent of moisture, as shown here. In the hardened plants the increase in total nitrogen and phosphorus is noticeable. The increase in phosphorus is in accord with the finding of greater amounts of calcium malophosphate in hardened plants by microchemical means.

The increase in the aminonitrogen of hardened plants is suggestive of the changes which occur in the proteins. Schaffnit (*44*) found the proteins from hardened plants to be more difficult to precipitate by freezing than those from greenhouse plants. He ascribed this to changes in the proteins resulting in the cleavage of the more labile "high molecular" forms. According to the above analyses, the aminonitrogen represents 35 per cent of the total nitrogen of the second fraction (F_2) in the hardened cabbages and about half that amount in the nonhardened plants, or 5.5 per cent of the total nitrogen in the first case and 2.5 per cent in the second case. Although this is a small change, it is not necessary nor probable that cleavage as far as to the amino acids should occur to prevent precipitation on freezing.

CHANGE OF HYDROGEN-ION CONCENTRATION OF A PLANT SAP ON FREEZING

INDICATION OF A CHANGE OF ACIDITY AS SHOWN BY COLOR CHANGES

One of the most striking of the changes which occur as a result of freezing is a change of color in plants which have colored leaves. Haas (*15*) has shown that the plant pigments act as an indicator of the cell-sap acidity. The change of reaction is best observed in such plants as coleus, which have the pigment in solution in the cell sap and not masked by chlorophyll. Using such a natural indicator is advantageous because it introduces no external factors. Besides, the range of hydrogen-ion concentration covered by the change of the pigment from red to blue is the same as that naturally occurring in the plant. Everyone has observed that various conditions affect the color of such pigments.

The color change on freezing is due to a change in the acidity of the plant juice. The juice of the variety of coleus used here reacts slightly acid to the pigment, as shown by the bright-red color. If coleus leaves are frozen, the reaction remains the same for a considerable time. If, now, the leaf is rapidly thawed out by being dipped into warm water, the red pigment at once changes to a decided blue color. This change can be observed almost in an instant, before oxidase activity can produce secondary changes. The rapidity of the change indicates a removal of the excess hydrogen-ion in much the same manner as by neutralization.

Similar changes of reaction can be produced in the white of egg or in cabbage juice on freezing. If methyl red is added to cabbage juice and the juice allowed to freeze, an increase in the acidity is indicated by the increasing red color of the indicator. Here the change is more or less masked by the chlorophyll. A similar change of acidity can be observed if the white of egg is frozen. It is necessary to use an indicator covering the proper range of acidity, in this case phenolphthalein, since white of egg is slightly alkaline. The white of egg is colored red by the phenolphthalein; but when frozen solid, the red color disappears, owing to an increase in the hydrogen-ion concentration. This latter case is to serve merely as a demonstration, since we are not especially concerned with such materials. The change of reaction here may be due to the high eutectic point of sodium bicarbonate and its separation in the solid phase on freezing. Certainly such changes in acidity as here shown have some influence on the precipitation of proteins and upon frost injury. It is well known from the work of numerous authors that the acidity of the medium is a factor of great importance in determining the state of hydrophilous colloids such as the proteins.

CHANGES OF REACTION ON PLASMOLYSIS

A change in reaction can be produced also by placing sections of coleus leaves in concentrated sugar solutions. The protoplasts of the trichomes are plasmolyzed, and the red color is deepened by the removal of water. After a time the color changes to blue, and at the same time the pigment begins to lake from the cell, indicating a change of permeability and the death of the tissue. If the plasmolysis is not carried too far nor allowed to continue until a change of color occurs, the cells regain their turgidity, and the pigment does not leach out. Hence, the duration of the reaction appears to be a factor in producing the injury, as well as the amount of change of acidity.

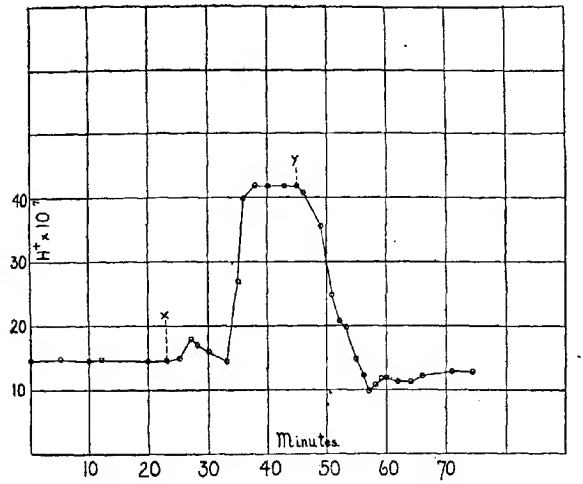


FIG. 1.—Graph showing change of the hydrogen-ion concentration of cabbage-leaf juice on freezing. Freezing at point *x*, thawing at point *y*.

POTENTIOMETRIC DETERMINATION OF THE CHANGES IN HYDROGEN-ION CONCENTRATION ON FREEZING

The change in acidity of a plant juice during freezing can be followed by the use of potentiometric methods. The potentiometer arrangement of Michaelis (*34*) was used. The measurements were made in a closed vessel provided with a dip hydrogen electrode essentially like that of Bovie (*3*) except that an exit tube was provided for the hydrogen.

As shown by the graph (fig. 1), the acidity of cabbage juice is increased by freezing. There is first a rise in the hydrogen-ion concentration, then a return to its original value, and then a very rapid rise to an acidity

much above that at the start. This value remains constant at the lower temperature and is directly proportional to the lowering of the freezing point. On thawing there is then a decrease in the hydrogen-ion concentration to a value less than the original, and then a return to the original value, or a value somewhat lower than the original. This change is almost paralleled by the increased acidity of a dilute solution of acid calcium phosphate, $Ca(H_2PO_4)_2$, on freezing. The graph for the plant juice differs from the latter, however, in the return to the original value at the start of the curve and in the depression past the original value on warming. This lag in the curve for the plant juice may be caused by removal of part of the hydrogen ions by proteins on freezing and the breaking of the combination on thawing out. If the juice is kept in the frozen condition for a longer time the final acidity is less than the original, evidently on account of some permanent combination of the excess hydrogen ion. This irreversible combination is indicated also by the color changes in coleus leaves.

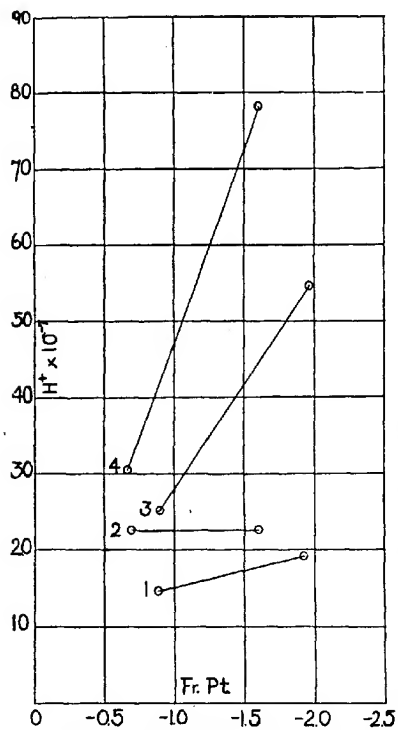


FIG. 2.—Graph showing increase in acidity with increasing depression of the freezing point on concentration of cabbage juice. 1 and 2, midrib juice; 3 and 4, juice from leaf minus midrib.

There are many objections to this method of measuring the increase of hydrogen-ion concentration on freezing, such as the melting of the ice by the stream of hydrogen, and the lack of uniformity in distribution of the unfrozen juice. To be free from these hindrances the following procedure was adopted:

The midribs and petioles of fresh cabbage leaves were cut out. This separation gave a more or less quantitative division of the tissue, although a large amount of vascular tissue was still left in the leaf. Juice was obtained from these portions by grinding them in a meat chopper and then expressing. Samples of a large quantity of juice were then taken for the determination of the freezing point and the acidity. The remainder was frozen, and the concentrated juice expressed from the ice. This concentrated juice was then sampled for freezing-point determinations and for acidity. The graphs in figure 2 show on the y-axis the increase in acidity on concentrating by the amount shown by the depression of the freezing point given on the x-axis. Graphs 3 and 4 represent the changes of acidity of juices expressed from cabbage leaves with the midrib removed. Graphs 1 and 2 give the acidity changes for juices expressed from the midrib and petiole alone. The original acidity of the juice from the midrib is a little less than that for the juice from the rest of the leaf, although the freezing point is practically the same. On concentrating, however, the acidity of the midrib juice does not increase nearly so rapidly as that of the juice from nonvascular tissue.

The concentrated juices were rediluted by adding the ice from which they had been expressed and then allowing the mixture to thaw. There is, of course, some loss in expressing, but this is comparatively small, as shown by the freezing point values. Petiole juice having a freezing point of $-0.88°$ C. and an acidity of $1.46\times10^{-6}H^+$ was concentrated to a freezing point of $-1.94°$ and an acidity of $1.92\times10^{-6}H^+$. All of the ice removed was saved and then remixed with the concentrated juice, giving the rediluted juice a freezing point of $-0.85°$ C. and an acidity of $0.96\times10^{-6}H^+$. In a similar manner the values for juice from the rest of the leaf were originally: Freezing point $-0.90°$, acidity $2.5\times10^{-6}H^+$; after concentration, freezing point $-1.96°$ C., acidity $5.45\times10^{-6}H^+$; and on rediluting, freezing point $-0.85°$ C., acidity $2.33\times10^{-6}H^+$. The concentration was the same for both samples as measured by the freezing point; still there was a much less change in the acidity of the juice from the petiole than from the nonvascular tissue. On rediluting, the acidity of the petiole juice is less than its original value, while that for the rest of the leaf is practically the same. The same amounts of acid were added to the same volume of juices expressed from the midrib and from the rest of the leaf, and then the vials were placed on ice. The petiole juice precipitated much more quickly than the other sample.

BUFFER EFFECT OF JUICE FROM MIDRIB AND REST OF LEAF

The juice from the petiole and midrib of cabbage has a lesser buffer effect than that from the rest of the leaf. This is shown on titration with *N*/10 sulphuric acid and *N*/10 sodium hydroxid (fig. 3). The number

of cubic centimeters of $N/10$ acid or $N/10$ alkali is plotted on the x-axis and C_H on the y-axis. The graph for midrib juice has a much steeper slope than the graph for juice expressed from the rest of the leaf, indicating a lesser ability to combine with the acid or alkali added. Precipitation was observed at the points marked x. This shows the point of precipitation on neutralization of the free hydrogen ion of both samples to be about $10^{-6.57}$ C_H. On adding acid the albumens of the petiole juice are precipated at a lesser hydrogen-ion concentration than that for juice of the rest of the leaf. To reach the higher hydrogen-ion concentration a considerably greater amount of acid is required. For instance, 50 cc. of

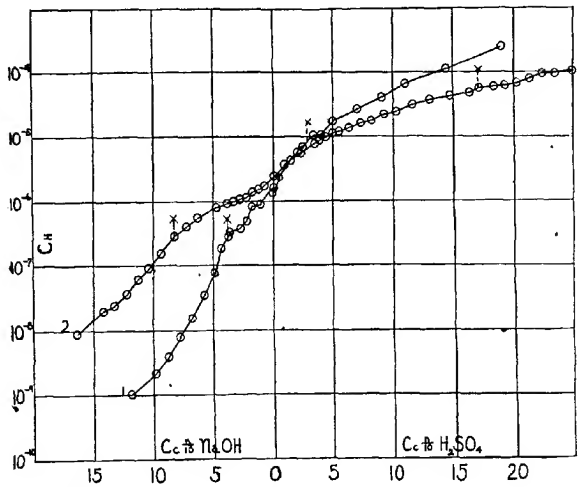


FIG. 3.—Titration graphs for cabbage juices: 1, juice from midrib: 2, juice from rest of leaf. Precipitation was observed at points marked "x." For explanation see text.

fresh petiole juice required 0.67 cc. of $N/1$ ortho-phosphoric acid (H_3PO_4) to change its acidity from 1.56×10^{-6} to 14.6×10^{-6} H^+, while the same amount of juice from the rest of the leaf required 1.48 cc. of $N/1$ ortho-phosphoric acid to change it from 2.1×10^{-6} to 14.6×10^{-6} H^+.

HYDROGEN-ION CONCENTRATION NECESSARY FOR NORMAL CONDITION OF THE PROTEINS

From figure 3 it is seen that there is an optimum hydrogen-ion concentration at which the proteins are held in solution in the cell sap. This extends from C_H $10^{-6.57}$ to about $10^{-4.3}$ when instantaneous precipitation of the proteins is taken as the measure. If a longer time is

allowed, lesser concentrations of acid precipitate the proteins. In fact, if phosphoric acid is added until the hydrogen-ion concentration is increased to the point to which it is increased by freezing, a precipitation of the protein occurs in about the time necessary to kill plants by freezing. However, changes in state much less than precipitation of the proteins may be sufficient to cause changes in permeability or even the death of protoplast.

DIFFERENCES BETWEEN THE MIDRIB AND THE REST OF THE LEAF

The foregoing data indicate that there is a difference between juices expressed from the midrib and from the rest of the leaf. The proteins in the midrib juice seem to be more easily precipitated by increase of acidity, and the juice has a lesser buffer effect. Simultaneous with the precipitation of proteins there is a decrease in acidity of the rediluted petiole juice to a value less than the original. The change of acidity is comparable to the conditions causing a change from red to blue in coleus leaves when they are thawed out. In juice from the rest of the cabbage leaf this does not occur to so great a degree. The irreversible precipitation of proteins is held to agree with the greater injury or stimulation of the cells of the midrib and those at the hydathodes than to those in the spaces between the veins, as shown by tumor formation both on freezing and on subjecting to ammonia fumes. It remains to be seen whether the cells of the midrib in plants generally may not have greater powers of regeneration than other cells; yet it is indicated by the more frequent regeneration from such cells in preference to other areas. It is suggested also that the point of regeneration may be determined by conditions similar to the above on stimulation in a similar manner by acidity increased through desiccation.

PRECIPITATION OF THE PROTEINS OF HARDENED AND NONHARDENED CABBAGE ON FREEZING

The precipitation of proteins on freezing was found by Schaffnit (*44*) to be greater in the case of juices expressed from greenhouse plants than from plants taken from the open in winter. Chandler (*5*) doubts that protein precipitation can be the cause of frost injury. However, his results show that protein precipitation occurs on freezing and in the case of hardened succulent plants to a lesser degree than in nonhardened plants. The differences in the precipitation of proteins given in his last report (*5*, *p. 186*) are small, but he suggests that they are within the range of experimental error. He does not state the relative hardiness of the plants used, and it is entirely possible that they were not thoroughly hardened so as to be frozen without injury.

The writer has determined the relative protein precipitation in hardened and nonhardened cabbages. Plants from the same lot were grown

part in a warm compartment of the greenhouse, part in a cold frame at temperatures usually above 0° C., but frequently low enough to freeze the plants stiff. The hardened plants were uninjured by an hour's freezing at −3°, while those from indoors were killed. Samples of juices were collected from the leaves of both hardened and nonhardened plants, using the same method for grinding and expressing in both cases. The juices were quickly frozen in Nessler tubes in a freezing mixture at −4° and kept at that temperature for two hours. Control samples were placed at once into ice water and kept there for the same time as the frozen samples. After two hours the frozen samples were quickly thawed in slightly warm water and then cooled to 0° as soon as all the ice had melted. All the samples were then placed in tubes and centrifuged together for eight minutes at high speed. Samples were withdrawn with a pipette to avoid disturbing the precipitates. The total nitrogen was determined by the Nitrogen Laboratory of the Bureau of Chemistry. On expressing the total nitrogen as proteins, it was found that 9.4 per cent of the original quantity of protein was precipitated from the hardened plants, and 31.2 per cent from the nonhardened plants by this period of freezing.

A change in state of proteins sufficient to allow these proportions to be precipitated by centrifuging the juices certainly bears some relation to frost injury. The lesser amount of precipitation in the case of juices from hardened cabbages indicates that this may account for the lesser injury to such plants.

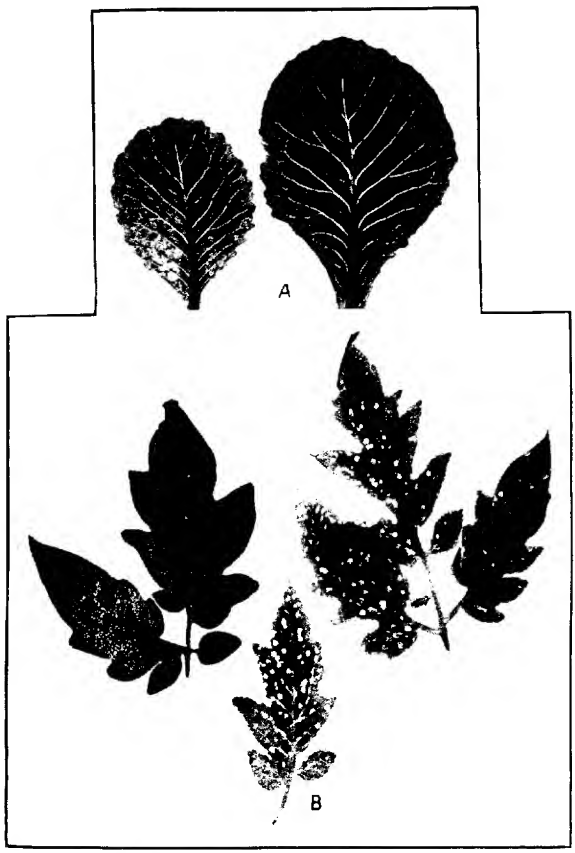
PRECIPITATION OF THE PROTEINS OF HARDENED AND NONHARDENED CABBAGES ON THE ADDITION OF ACID

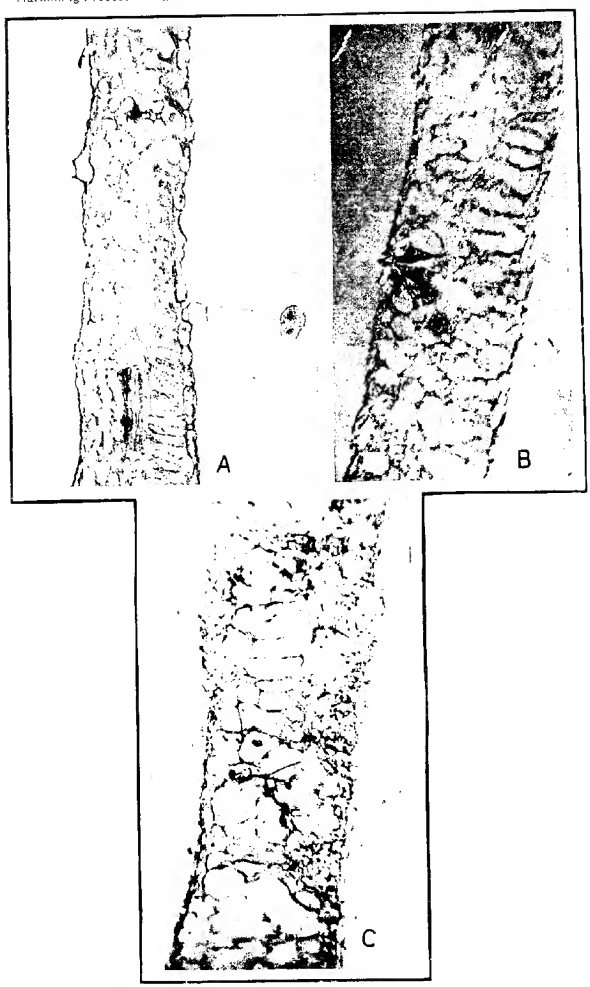
Juices were obtained in the same manner as before from hardened and nonhardened cabbages. To 50 cc. samples a sufficient quantity of acid was added to increase the hydrogen-ion concentration by approximately the amount by which it was increased by freezing at −3° C. This quantity was found by previous experiments to be 5 cc. of *N/10* sulphuric acid for 50 cc. of juice from nonhardened plants. The juices were kept in ice water for an hour, together with controls. The samples were then treated as in the previous experiment. It was found that this quantity of acid precipitated 11 per cent of the original quantity of proteins in the juice from hardened plants and 44 per cent in the juice from nonhardened plants. This indicates that the proteins are more easily precipitated by increase of the hydrogen-ion concentration in the juices of nonhardened plants than of hardened plants. This sensitiveness of the proteins to the addition of acid is closely paralleled by their sensitiveness to freezing.

PLATE 7

A.—Injected areas of cabbage leaves photographed by transmitted light immediately after freezing.

B.—Injected areas of tomato leaves photographed by transmitted light immediately after freezing. The leaf at the left shows the mottled appearance which occurs after a few days.


A
B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C

PLATE 8

A.—Tomato leaf showing the collapse of the palisade in the frozen areas. Note the normal condition of the cells at the right of the trichome, in which no ice formation occurred.

B.—Crystals of calcium malo-phosphate in the injected areas of cabbage leaves photographed by polarized light.

C.—Young tumor of cabbage leaf three days after freezing, showing large nuclei and multinucleate cells.

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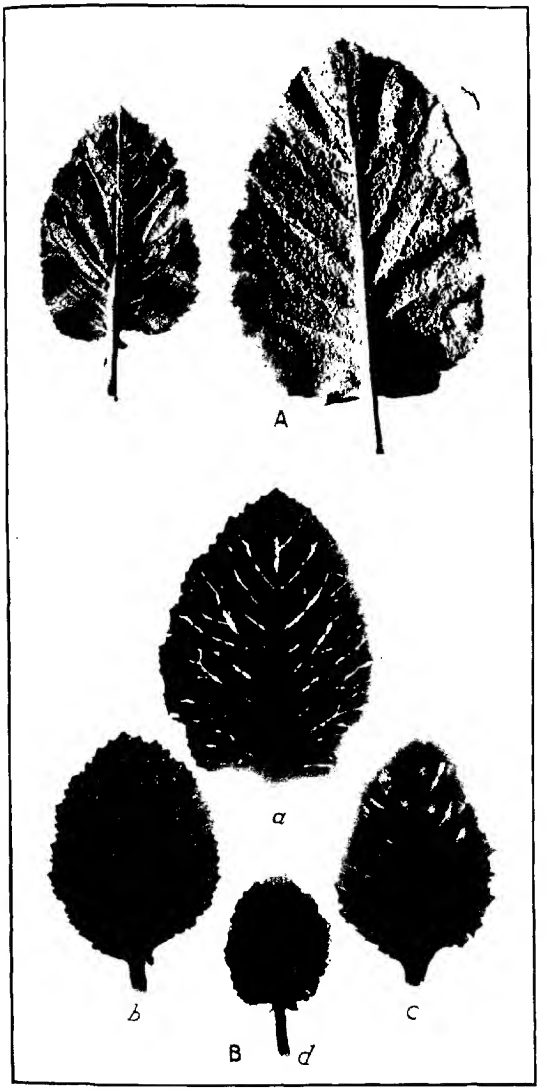
PLATE 9

A.—Distribution of the intumescences on cabbage leaves. In the leaf at the right nearly all of the leaf cells show renewed growth.

B.—Intumescences on cabbage leaves photographed by transmitted light. a, b, d show the occurrence along the veins, and c the development at the hydathodes.


A
a
b
c
B
d

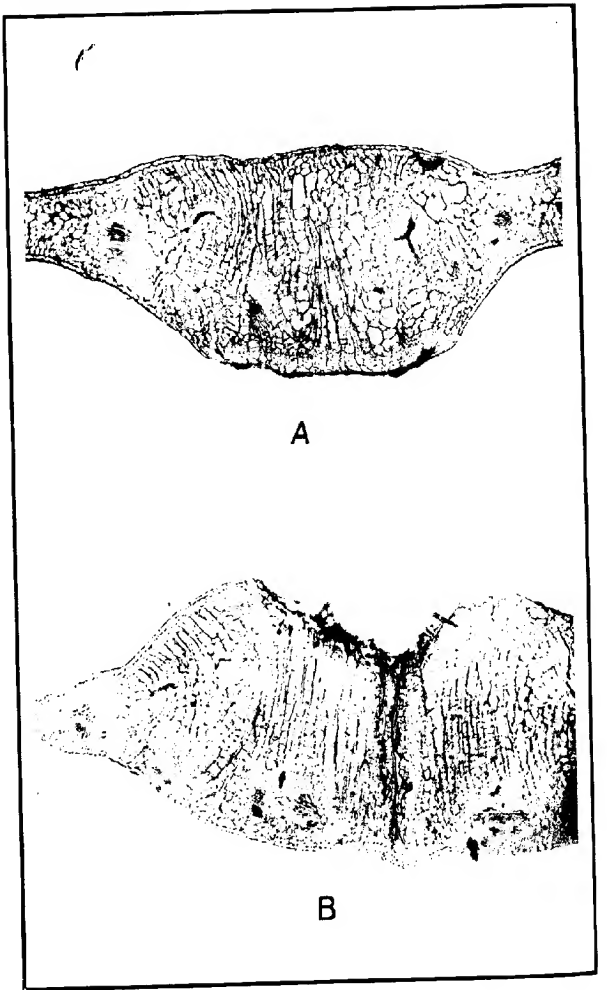

A
B

PLATE 10

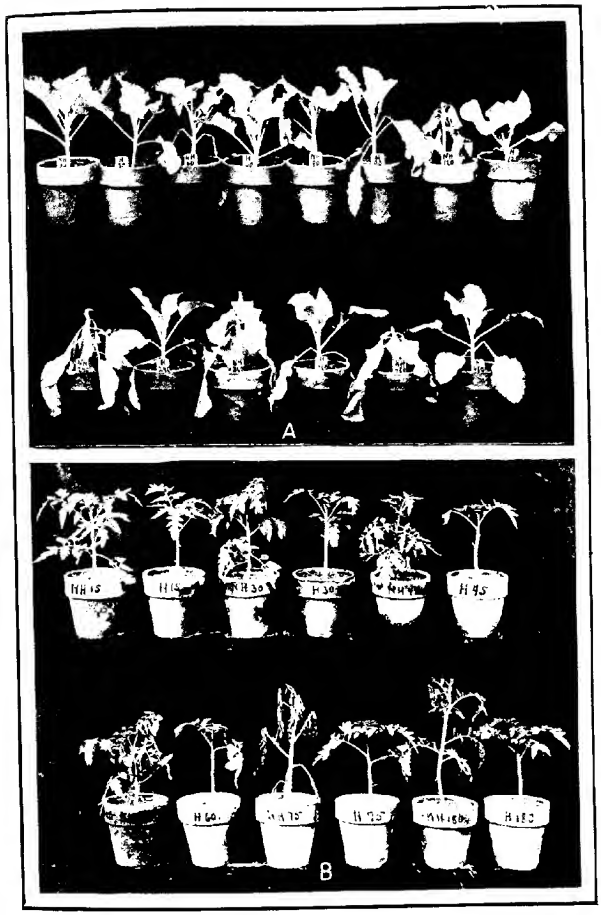
A.—Section of cabbage tumor after seven days, showing the chains of cells beginning at the epidermis and areas of small cells in active division in the center of the leaf.

B.—Section of cabbage tumor after two weeks' growth. The epidermis has been broken at the top, and infection may occur. Some sclerenchymatous cells with thick lignified walls have developed in the center of the tumor.

PLATE 11

A.—Cabbage plants showing the relative injury to hardened (H) and nonhardened (NH) plants after exposure for the time given on the label in minutes to a temperature of -3° C. Breaking of the petiole while frozen caused some of the lower leaves of the hardened plants to droop; otherwise these leaves were uninjured.

B.—Tomato plants showing the relative injury to plants from the greenhouse (NH) and from coldframes (H) after exposure for the time given on the labels in minutes to -1.5° C. The plants from the coldframes were not frozen. The young leaves show the injury first.


A
B

DISCUSSION OF RESULTS

Haberlandt (*16*) found that a growth similar to that of the tumor cells of cabbage occurs in the storage cells which lie next to the phloem tissue in small pieces cut from potato tubers. Growth occurs in pieces 0.5 by 1 by 1 mm. only when phloem cells are present. Xylem cells are not necessary. Haberlandt stated that growth does not occur in such cells because they are physiologically different from cells farther from the phloem bundles. Neither is growth due to nutrient materials coming from the phloem; nor is it due to the phloem cells giving off substances which counteract the lethal overstimulation of the cells by wound stimulus. He attributed the growth to the excretion of a growth-producing enzym (*Wuchsenzyme*) by the phloem cells and stated that the companion cells rather than the sieve tubes are responsible for it.

In any such case or in freezing it is difficult to establish just what wound stimulus consists in. In the case examined in this investigation it appears that at least a part of the wound stimulus can be accounted for by the change of the hydrogen-ion concentration on freezing and by the consequent precipitation of proteins. This offers a tangible thing to which wound stimulus can be ascribed. It is possible also that other wound stimuli may be caused in a similar manner by desiccation. In nearly all such cases it appears that a stimulus which causes growth on moderate application will cause death on overstimulation of the cells. Thus, the first layers of a cut surface of potato die, and phellogen formation is taken up by the lower layers. Similarly, cabbage cells are either stimulated to growth or killed outright, according to the degree of freezing. The greater stimulation of the tissue about the veins to form tumors on freezing is possibly due to the fact that such cells are more easily injured than the other cells of the leaf, and thus receive a stronger stimulus. The easier precipitation of the proteins in juices expressed from the midrib than from the rest of the leaf indicates such a physiological difference.

Cases of renewed growth or regeneration, such as shown by Klebs (*19*) and Miehe (*35*) to be produced on plasmolysis, have usually been ascribed to removal of the cells affected from the influence of those about them—that is, to a removal of the correlation effect. It has been reported by Klebs (*19*) that growth after plasmolysis generally occurs only in the algæ. Miehe (*35*) states that in such plasmolyzed cells only the living continuity is destroyed. This statement is taken to refer to the plasmodesmen. It has been shown by Gardiner (*10*) and others (*11*) that there is a continuity of protoplasm between adjacent cells. Gardiner (*10*) found that these threads of protoplasm were not broken in every case during plasmolysis, but fine strands could be observed running out to the cell wall. Mangham (*25*, *26*) ascribes to the plasmodesmen an important function in conducting sugar from cell to cell. Czapek (*7*)

showed that conduction of food materials occurred after the plasmolysis of these conducting cells. The protoplasmic connection between cells is, therefore, not necessarily broken by plasmolysis, according to the evidence above cited. Consequently it is necessary to assume that in some manner these strands are killed on plasmolysis if the growth stimulus is attributed to the removal of correlation factors and the breaking of protoplasmic connection between the cells.

It is entirely possible that the strands running to the cell wall in plasmolysed cells may remain alive, since they are scarcely thinner than the plasmodesmen themselves. In this case the "living continuity" is not broken. It is equally possible that they are killed, and in such case death may be due to changes in the state of the protoplasm constituents owing to changed acidity and an increase in the concentration of the salts. If these threads are killed on plasmolysis of the cells, equal changes can be expected to occur on the surface of the protoplast. It was Chandler's idea that—

> killing from cold is more likely a mechanical injury due to the withdrawal of water from the protoplasmic membrane than an injury resulting from a precipitation of proteins.

The effect of plasmolysis on the regeneration of plasmolyzed cells can then be ascribed also to this change in the membrane as well as to the removal of the correlation effect. If growth is due to the removal of the correlation effect through plasmolysis of the cells on freezing then one would expect the plasmolyzed cells of the hardened cabbages to be stimulated to growth in the same manner as those of nonhardened cabbages. But this does not occur; consequently the renewal of growth in the cells can not be ascribed to the breaking of the living continuity and removal of the correlation effect in the case of the cabbage. The lack of growth in the cells of hardened cabbages is evidently due to their inability to withstand the concentration of the cell sap and increased acidity without injury.

It appears that in the process of hardening there are changes which occur in the proteins. To these changes the greatest effect of the hardening is to be ascribed. The changes are cleavages to simple forms if the increase of amino acids on hardening of the plants is taken as an indication. The simple forms of the proteins are not so easily precipitated by freezing or by the addition of acid as the more complex forms. The increase of acidity of a plant juice on freezing is accompanied by an increase in the concentration of the salts. Both conditions are favorable for protein precipitation.

The surface layer is the most exposed part of the plasmolyzed protoplast. It is therefore probable that the greatest changes in aggregation of the proteins occur there.

The change in color of coleus leaves after freezing indicates that

gen-ion concentration is decreased on thawing. This is probably the case in the frozen cells of the injected areas of nonhardened cabbages. In those cells which survive one would then expect to find a slightly decreased acidity. No indicator covering the proper range of acidity has been found which will penetrate the cabbage cells in sufficient concentration to show a color deep enough not to be masked by the chlorophyll. However, such a change is indicated by the change of the injected spots on coleus leaves from red to blue. The effect of the decreased acidity may be to cause greater activity of the peroxidase present or to allow its accumulation. Blackening occurs in the areas of *Aucuba japonica* injected by freezing, indicating an increased activity of the oxidizing enzyms. Krasnosselsky (*22*) reports an increase in the concentration of the oxidizing enzyms due to wound stimulus. It is known that the oxidizing enzymes are destroyed quite rapidly at the hydrogen-ion concentration shown by cabbage leaves and that a decrease in acidity favors their action.

SUMMARY

(1) The first indications of frost injury to succulent plants were observed in the appearance of injected areas over the leaf surface. These injected areas are caused by the withdrawal of water from the protoplast and the displacement of air in the intercellular spaces. Inoculation of the undercooled leaf tissue from ice formed on the surface is generally the cause of the local freezing. Wax on the leaf surface prevents the inoculation of the undercooled tissue and thus prevents injury from freezing.

(2) Frozen cells in the leaves of cabbage, bryophyllum, salvia, and lettuce were found to be stimulated to growth and to produce tumors similar to those shown in pathological conditions, but without the presence of bacteria. Frozen spots on the leaves of tomato, coleus, geranium, and a number of other plants did not receive a growth stimulus as in the former cases, but were killed by the freezing. This local killing of the tissue gives such leaves a spotted appearance.

(3) The peroxidase content of the intumescences of cabbage induced by freezing was found to be much greater than that for normal leaf tissue. A decrease in the hydrogen-ion concentration may occur in such cells, and this condition may allow greater activity or accumulation of the respiratory enzyms, especially peroxidase.

(4) It is suggested that the growth stimulus in frozen cells is not due to the removal of the correlation effect on plasmolysis of the cells, but is to be ascribed to a partial precipitation of the proteins of these cells. This precipitation results in an increase in the permeability of the cells to water, and in the ability of the cells to hold sugars.

(5) The apparent osmotic pressure on plasmolysis of the tumor cells was found to be less than that for normal mesophyll cells. However, the tumored areas of the leaf do not freeze more readily than the other areas.

(6) The principal effect of the hardening process for cabbages is a change in the constituents of the protoplasm which prevent their precipitation as a result of the physical changes incident upon freezing. The proteins are changed to forms which are less easily precipitated. This is indicated by an increase in the amino-acid content of the cabbage plants on hardening.

(7) The factors which produce protein precipitation on the freezing of a plant juice are held to be principally the increase in the hydrogen-ion concentration and the increase in the concentration of the salts. The latter factor is held to be insufficient to cause precipitation except under the conditions of a changed acidity. Cabbage plants were found to become resistant to a half-hour's freezing at $-3°$ C. after exposure to $+3°$ for five days. During this time the carbohydrate changes were slight. Hence, the prevention of protein precipitation by sugar accumulated during hardening is not sufficient to account for the resistance of hardened plants to freezing.

(8) The proteins of the midrib of cabbage leaves are precipitated more readily than those from the rest of the leaf. This is considered to be due to physiological differences between vascular tissues and the other tissues of the leaf.

(9) In juices of nonhardened and hardened cabbages the proteins of the former were found to be precipitated to a greater degree by freezing than those of the latter. The percentage of precipitation for such juices on freezing is closely paralleled by the relative precipitation on the addition of acid.

(10) The greatest changes induced by freezing are supposed to occur n the outer portions of the protoplast since this is most exposed on plasmolysis.

(11) The effects of desiccation, freezing, and plasmolysis are considered to be similar, in that all these processes cause changes in the hydrogen-ion and salt concentrations.

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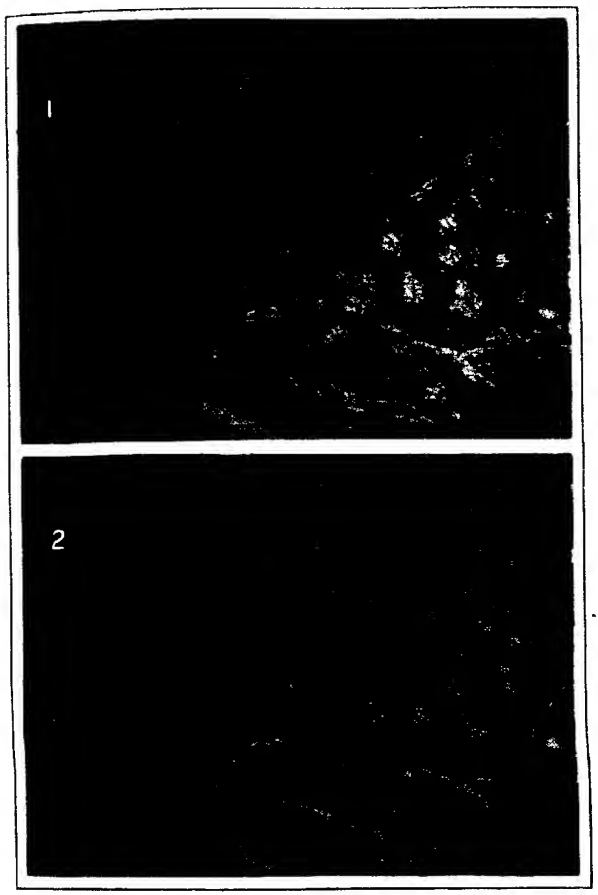
PLATE A

1.—A cabbage leaf showing the chlorophyll distribution in the intumescences.

2.—A cabbage leaf showing the comparative peroxidase reaction given in the tumor and leaf cells with tetramethylparaphenylenediamine.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CHEMISTRY OF SWEET-CLOVER SILAGE IN COMPARISON WITH ALFALFA SILAGE

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INTRODUCTION

In pursuing chemical studies in making alfalfa silage, previously reported,[1] there were certain phases which seemed to warrant further investigation. In outlining the plan for this additional work it seemed desirable to broaden the investigation by including sweet clover. As in the previous investigations, milk bottles were used as containers.

PLAN OF THE WORK

After the alfalfa (*Medicago sativa*) or the sweet clover (*Melilotus* spp.) had been cut, it was allowed to wilt in the sun for about two hours. The alfalfa was cut in one-tenth bloom, the sweet clover just before bloom. The alfalfa was of average size, fine quality, first cutting. The sweet clover was very rank and had a high moisture content. After wilting, the material was brought to the laboratory and passed through a small feed cutter. Alfalfa was used alone. The sweet clover was used alone and also with ground corn in the proportion of 1 to 10. Each bottle was weighed before filling. All were packed full, closed with corks which were wired, and sealed with wax such as is used in closing desiccators.

Three bottles, one of each kind, were opened each day for the first week, then every other day for the second week, then each week for the next four weeks, and finally each month as long as needed. On opening, the silage was judged as to color and grade. All the bottles, without exception, had first-class silage. In this study an effort was made to obtain as nearly as possible the same grade of silage in all the bottles. From our previous work the conditions for obtaining this result were known.

LOSS OF MATERIAL

As soon as a bottle was opened the content was weighed. The weight of the material put into the bottle, about 700 gm., had been obtained at the time of filling. From these figures were calculated the loss of material during silage making. The figures obtained are given in Table I.

[1] SWANSON, C. O., and TAGUE, E. L. CHEMICAL STUDIES IN MAKING ALFALFA SILAGE. *In* Jour. Agr. Research, v. 10, no. 6, p. 275–292. 1917.

TABLE I.—*Loss of weight, in grams, of various kinds of silage*

Age of silage.	Alfalfa alone.	Sweet clover alone.	Sweet clover and corn-chop.	Age of silage.	Alfalfa alone.	Sweet clover alone.	Sweet clover and corn-chop.
Days.				*Days.*			
1	2.0	6.0	1.0	13	4.0	4.0	6.0
2	5.8	6.0	.....	15	6.0	5.0	9.0
3	6.8	6.5	6.0	17	9.8	6.6	10.5
4	5.0	8.0	7.3	21	7.0	8.1	39.0
6	8.0	7.0	6.0	29	7.1	7.6	9.8
7	9.0	.....	.....	43	8.3	23.3	1.6
8	8.0	6.0	.....	64	7.7	10.5	9.0
10	6.0	7.0	5.8	98	7.0	8.6	7.0

These results show that the losses, with a few exceptions, were approximately 1 per cent. The greater part of the larger losses was silage juice. The gas generated in the bottle would push out the stopper slightly, resulting in loss. Since the total loss is so small, the losses due to fermentation are insignificant.

MOISTURE CONTENT

The moisture content in the silage was determined on 100-gm. samples, using the material in the condition in which it was taken from the bottles. The moisture percentages are given in Table II.

TABLE II.—*Percentage of moisture in alfalfa silage*

Age of silage.	Alfalfa alone.	Sweet clover.	Sweet clover and corn-chop.	Age of silage.	Alfalfa clover.	Sweet clover.	Sweet clover and corn-chop.
Days.				*Days—Contd.*			
0	63.2	77.5	71.2	15	64.5	77.0	71.4
1	63.1	77.3	71.2	17	64.1	75.9	72.2
2	64.3	75.4	71.4	21	63.6	77.0	72.7
3	62.2	75.9	71.9	29	62.1	77.1	72.5
4	63.7	77.3	73.0	43	62.1	76.1	72.6
6	64.6	76.7	71.2	64	62.4	77.4	72.9
7	64.8	.....	.....	98	62.9	77.5	73.2
8	64.6	76.5	73.3				
10	64.9	77.4	72.1	Average	63.6	76.5	72.1
13	64.9	77.8	71.7				

The general agreement of these figures for each kind of silage shows that the material in the bottles was fairly uniform.

METHOD OF MAKING THE WATER EXTRACT OF SILAGE

When each bottle was opened, 100 gms. of silage were weighed into a quart Mason jar and 430 cc. of carbon-dioxid-free water added, so as to make the total moisture content very nearly 500 cc. Thus, each 5 cc.

represented the extract from 1 gm. of silage. This was on the assumption that the average moisture content was 70 per cent. This method of procedure involved a slight error which is without significance in this work. The jar was closed and placed on a shaking machine for two hours, after which the material was strained through a linen cloth into a 500-cc. centrifuge cup, and centrifuged for five minutes at the rate of 2,400 revolutions per minute. The average relative force, times gravity, is 1,344 at this speed, according to the statement of the manufacturer. This gave a clear but dark-colored supernatant liquid. In order not to stir up the sediment in the bottom of the cup, it was necessary to allow the centrifuge to come to a stop without using the brake. Because of the presence of some light particles which would not settle to the bottom, it was necessary to filter on folded filters. This filtrate was then used for the following determinations:

(1) Acidity by titrating to phenolphthalein.

(2) Acidity by using the hydrogen electrode.

(3) The nitrogen in amino form, by titrating in the presence of formaldehyde using thymolphthalein as indicator.

(4) The nitrogen in amino form by titrating in the presence of formaldehyde to a certain hydrogen-ion concentration, using the hydrogen electrode.

(5) Total nitrogen in the water extract.

(6) Total nitrogen in water extract not precipitated by phosphotungstic acid.

An attempt was made to determine nitrogen in the water extract by Stutzer's method, but the amounts were so small that differences were within the analytical error.

METHOD OF MAKING THE ALCOHOLIC EXTRACT OF SILAGE

When each bottle was opened, 100 gms. of the silage were weighed into a quart Mason jar and 250 cc. of 95 per cent alcohol added. The jar was then sealed and allowed to stand till a convenient time for doing the work. This was necessary, as the work on the water extract had to be finished at once, and occupied the available time. On the basis of total moisture in the silage, previously determined, enough carbon-dioxid-free water was added to the jar to make the volume 500 cc. This would make the percentage of the alcohol about 50. After adding this water the jar was sealed, shaken, and allowed to stand for an hour, when the material was strained through linen cloth. From this point the precedure was the same as with the water extract, and the same chemical determinations were made.

ACIDITY IN THE WATER AND THE ALCOHOLIC EXTRACTS OF SILAGE, TITRATING TO PHENOLPHTHALEIN

Twenty-five cc. of the water or the alcoholic extract, representing 5 gms. of silage were pipetted into a 500-cc. Jena Erlenmeyer flask, and 200 cc. of carbon-dioxid-free water were added. One cc. of phenolphthalein as indicator was used. The extract was then titrated to a faint pink with *N/20* sodium hydroxid. The results obtained are presented in Table III.

TABLE III.—*Acidity in water and alcoholic extracts of silage*

[Results expressed as cubic centimeters of *N/20* sodium hydroxid on the extract from 5 gm. of silage titration with phenolphthalein as indicator]

Age of silage.	Alfalfa alone.		Sweet clover alone.		Sweet clover and corn chop.	
	Water.	Alcohol.	Water.	Alcohol.	Water.	Alcohol.
Days.						
0	3.3	7.7	2.8	3.6	3.3	3.8
1	9.0	11.7	4.6	6.1	5.3	5.7
2	10.4	12.0	3.4	7.2	5.3	6.9
3	11.4	12.9	6.3	8.7	5.6	7.6
4	9.1	13.5	8.7	10.1	8.2	10.0
6	10.6	14.9	8.0	9.4	9.0	10.9
7	14.4	15.4	....	....	....	....
8	11.5	15.0	6.4	9.9	13.2	14.8
10	13.4	16.9	11.6	12.5	9.3	13.2
13	17.8	19.0	11.3	12.8	13.2	16.1
15	18.5	19.9	10.3	13.6	14.3	17.3
17	16.6	20.8	12.2	14.7	14.8	17.3
21	18.1	22.4	13.8	15.5	16.6	20.0
29	20.8	25.2	13.0	16.5	17.0	21.0
43	26.6	28.4	11.7	16.5	16.8	22.4
64	25.7	30.4	12.6	17.7	18.9	25.2
98	26.7	....	12.8	18.5	18.6	26.3

The figures in Table III show the following results:

(1) The acidity in the alcoholic extract appears to be uniformly greater than in the water extract. The silage made from sweet clover alone and from sweet clover plus corn meal show the same relative differences in the acidity of the alcoholic and water extracts as the silage made from alfalfa alone. That the alcoholic extract from corn silage will give a larger percentage of acidity than will the water extract when phenolphthalein is used as the indicator has been shown in a previous publication from this laboratory.[1] A suggestion was reported in that journal that the greater acidity of the alcoholic extract is due to fatty acids liberated by lipases which are active in silage formation. That this explanation is probably not correct will be shown in a subsequent part of this paper.

[1] SWANSON, C. O., CALVIN, J. W., and HUNGERFORD, Edwin. ACIDITY IN SILAGE: METHOD OF DETERMINATION. Jour. Amer. Chem. Soc., v. 35, no. 4, p. 476-483. 1913.

(2) The acidity of matured silage made from alfalfa alone is twice that of matured silage made from sweet clover alone, as determined in the water extracts. As determined in the alcoholic extract, the acidity is approximately one-third greater.

(3) The comparatively smaller acidity of the silage made from sweet clover may be partly due to the high moisture content. In a previous investigation[1] it was found that silage made from freshly cut alfalfa had a lower percentage of acidity than that made from wilted alfalfa. While the sweet clover used in the present investigation was wilted, it still had a large moisture content.

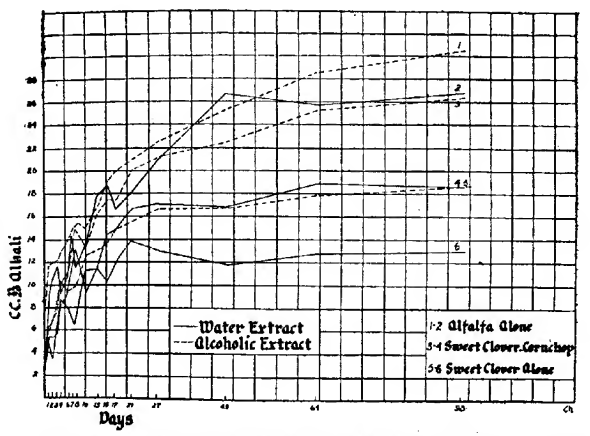

FIG. 1.—Graphs showing the acidity in water and alcoholic extracts of silage; obtained by colorimetric titration with phenolphthalein.

(4) The acidity of the water-extract silage from alfalfa alone is the same as the acidity of the alcoholic extract of silage from sweet clover plus corn meal.

(5) When corn meal was added to sweet clover, the acidity was increased about 50 per cent. This is in harmony with results obtained in a previous investigation[2] with adding corn meal to alfalfa in making silage.

(6) About two-thirds of the total acidity is developed in the first 15 days. The maximum acidity is reached in about 40 to 60 days. (See fig. 1.)

[1] SWANSON, C. O. and TAGUE, E. L. OP. CIT., p. 281.
[2] SWANSON, C. O., and TAGUE, E. L. OP. CIT., p. 282.

TITRATION TO A CERTAIN HYDROGEN-ION CONCENTRATION WITH THE HYDROGEN-ELECTRODE APPARATUS

The general arrangement of the apparatus for determining the hydrogen-ion concentration by the use of the hydrogen electrode was that given by Hildebrand.[1]

Our outfit contains the following pieces: One Weston D. C. milli-voltmeter and multiplier; one Kohlrausch slide wire bridge with extension coils; one Leeds and Northrup No. 2,500 type R. galvanometer with lamp and scale; two Edison storage batteries. The calomel and hydrogen electrodes were made according to the directions given by Hildebrand.

For the first part of this work neither this bridge nor the galvanometer were available. The bridge used was a straight-wire bridge such as is commonly found in physics laboratories. Instead of the galvanometer we used the Lipmann electrometer and reading telescope.

The galvanometer with the lamp and scale, as well as the Kohlrausch bridge, are found exceptionally satisfactory for this class of work.

As a source of hydrogen we have found that made by the electrolytic process very satisfactory. This can be bought compressed in iron cylinders. As a precaution against impurities the gas is washed in a train of alkaline pyrogallic acid, potassium permanganate, mercuric chlorid, and carbon-dioxid-free water. The hydrogen made from aluminium and sodium hydroxid was found just as satisfactory, but more expensive as to materials and labor.

METHOD OF DETERMINING THE TITRABLE ACIDITY BY THE USE OF THE HYDROGEN ELECTRODE

Twenty-five cc. of the water or alcoholic extract were pipetted into a wide mouth 250-cc. flask. The calomel and the hydrogen electrodes were inserted through a rubber stopper which fitted the mouth of the flask. As the lower end of the hydrogen electrode was bell-shaped, it was necessary to use a partially split stopper. Through a third hole in the stopper the tip of a burette was inserted so that the N/20 sodium hydroxid could be added without exposing the contents of the flask to the air. Hydrogen gas was then allowed to bubble through the solution with frequent shaking and the bridge adjusted until equilibrium was obtained—that is, until no current flowed through the galvanometer. At this point the reading of the milli-voltmeter was noted. Next, N/20 sodium hydroxid was added from the burette slowly with frequent shaking. It was desired to measure the amount of alkali necessary to add in order to obtain three different hydrogen-ion concentrations: $P_H 7$,

[1] HILDEBRAND, J. H. SOME APPLICATIONS OF THE HYDROGEN ELECTRODE IN ANALYSIS, RESEARCH AND TEACHING. *In* Jour. Amer. Chem. Soc., v. 35, no. 7, p. 847-871, 15 fig. 1913.

the true neutral point; P_H8.3, the point of color change for phenolphthalein; and P_H9.3, the point of color change for thymolphthalein. The *N/20* sodium hydroxid was added until equilibrium was reached for each point and the total cc's noted. When the last point was reached, 25 cc. of formaldehyde solution which had previously been neutralized to a hydrogen-ion concentration of P_H9.3 was added and *N/20* sodium hydroxid added again until the potential indicated a concentration of P_H9.3. This last operation gave the figures for calculating the titrable nitrogen.

The number of cubic centimeters of *N/20* sodium hydroxid necessary to titrate to the true neutral point, P_H7, also to the color change of phenolphthalein P_H8.3, and to the color change of thymolphthalein, P_H 9.3 are given in Table IV.

TABLE IV.—*Quantity, in cubic centimeters, of N/20 sodium hydroxid necessary to titrate to three different hydrogen-ion concentrations, P_H 7 P_H 8.3, and P_H 9.3*

[Each sample represents the extract from 5 gm. of silage]

TITRATION TO P_H7

Age of silage.	Alfalfa alone.		Sweet clover alone.		Sweet clover and corn-chop.	
	Water.	Alcohol.	Water.	Alcohol.	Water.	Alcohol.
Days.						
0	2.3	4.2	1.0	1.9	1.4	2.2
1	6.0	5.3	2.7	2.6	2.9	2.5
2	6.3	7.5	3.6	3.9	3.5	3.6
3	6.1	7.8	4.6	5.2	4.3	4.1
4	4.8	7.9	6.9	6.0	8.0	6.0
6	5.7	7.5	10.3	5.4	8.0	6.2
7	9.6	7.6				
8	8.1	9.6	7.0	5.7	10.2	9.6
10	9.4	10.4	10.2	8.3	9.6	8.4
13	10.0	11.1	9.8	8.6	12.0	11.1
15	12.0	12.0	8.6	9.0	12.1	12.5
17	11.6	13.0	10.6	10.6	11.7	13.0
21	13.1	14.2	11.8	10.8	15.2	14.9
29	16.0	17.0	11.5	11.2	15.0	15.1
43	19.0	21.3	10.0	11.2	14.7	16.7
64	19.4	22.6	10.5	11.7	16.5	19.0
98	22.0	24.7	10.1	13.0	17.3	20.1

TABLE IV.—*Quantity, in cubic centimeters, of N/20 sodium hydroxid necessary to titrate to three different hydrogen-ion concentrations, $P_H 7$ $P_H 8.3$, and $P_H 9.3$*—Continued

TITRATION TO $P_H 8.3$

Age of silage.	Alfalfa alone.		Sweet clover alone.		Sweet clover and corn-chop.	
	Water.	Alcohol.	Water.	Alcohol.	Water.	Alcohol.
Days.						
0	3.3	6.3	2.9	3.2	3.1	3.3
1	8.6	8.5	5.1	4.3	6.2	4.9
2	11.0	11.0	6.7	6.1	6.9	5.4
3	11.1	11.1	8.1	7.4	8.0	6.5
4	8.4	11.3	10.4	8.4	11.5	8.2
6	10.1	11.3	13.0	7.7	11.0	8.8
7	14.2	11.2	.....	.....	.....	.....
8	11.8	13.2	9.3	8.0	13.0	12.4
10	13.5	14.0	12.5	11.1	12.6	11.1
13	13.5	14.8	12.2	11.4	14.6	14.2
15	15.0	15.8	11.5	11.7	14.8	15.1
17	14.7	16.8	13.3	13.6	14.4	15.6
21	21.6	18.1	15.1	14.0	19.8	18.0
29	21.8	21.5	14.5	14.3	18.5	18.2
43	26.0	25.7	13.8	14.1	18.7	20.1
64	26.9	26.4	14.0	14.7	21.4	23.0
98	26.5	29.2	12.7	15.9	21.8	23.7

TITRATION TO $P_H 9.3$

0	5.1	9.4	6.3	5.2	5.0	5.2
1	12.2	14.3	11.3	7.9	10.9	8.5
2	18.8	17.3	12.2	10.5	12.2	9.9
3	19.0	18.0	13.5	12.1	13.1	11.7
4	15.0	18.5	17.4	14.4	15.2	13.8
6	16.8	19.2	17.8	12.8	16.4	15.0
7	19.6	19.0	.....	.....	.....	.....
8	17.7	23.5	14.3	13.7	18.7	18.2
10	22.4	23.2	17.7	17.4	18.0	17.7
13	21.3	24.3	17.7	17.4	22.3	21.4
15	23.7	25.0	19.3	18.4	24.6	21.5
17	27.5	27.1	20.1	20.8	24.0	23.2
21	31.8	28.2	22.6	21.4	28.7	25.8
29	33.5	33.4	24.1	22.6	26.0	26.7
43	36.1	38.0	20.8	22.2	26.0	28.0
64	37.9	38.8	21.1	23.5	28.5	31.8
98	39.1	42.5	19.5	24.4	26.9	32.7

The data presented in Table IV show the following results:

(1) The acidity values obtained by the electrometric titration were practically the same for the alcoholic extract as for the water extract. In this respect it differs fundamentally from the colorimetric titration.

(2) Titrating to the true neutral point, $P_H 7$, gave a much lower acidity value than the colorimetric titration (fig. 2). Compare Tables III and IV, part 1.)

(3) Titrating to the hydrogen-ion concentration, $P_H 8.3$, or the point of color change for phenolphthalein, gave practically the same acidity value as was obtained in the water extract with colorimetric titration. (Compare Tables III and IV, part 2.)

(4) Titrating to the hydrogen-ion concentration $P_H 9.3$, or the point of color change for thymolphthalein, gave an acidity value about twice that obtained by titrating to the strictly neutral point, $P_H 7$.

(5) The acidity value of both the water and the alcoholic extracts obtained by titrating to the hydrogen-ion concentration $P_H 8.3$ agreed substantially with the value obtained in the water extract by the colorimetric titration. (See Table III.)

(6) The hydrogen-ion concentration of the water extract and the alcoholic extract agree substantially for the three points determined.

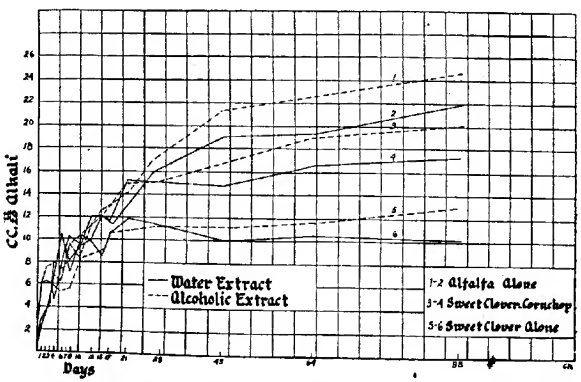

FIG. 2.—Graphs showing the quantity, in cubic centimeters, of alkali used in water and alcoholic extracts of silage; obtained by electrometric titration to $P_H 7$.

(7) These results show that the acidity value in an alcoholic extract of silage obtained by titrating to the color change of phenolphthalein are high in comparison with the true acidity value—that is the hydrogen-ion concentration. The results obtained by the colorimetric titration on the water extract correspond more nearly to the true acidity value than those obtained on the alcoholic extract. It has previously been suggested that the higher acidity value of the alcoholic extracts are due to the presence of fatty acids which are soluble in alcohol, but not in water. If this was the case the hydrogen-ion concentration of the water extract should be less than that of the alcoholic extracts. But the data show that this is not so. The higher acidity value obtained in the alcoholic extracts when the colorimetric titration is used is probably due only to the masking of the end point. The alcoholic extract contains more of

highly colored substances in solution than the water extract. That this was the case was suspected in our former investigation. Concerning this we said [1]:

> The figures for acidity are probably large. . . . . The extract is highly colored, and the end point is not easily read.

DIFFERENCES IN TITRATING TO THE HYDROGEN-ION CONCENTRATION $P_H8.3$ IN COMPARISON WITH P_H7; ALSO $P_H9.3$ IN COMPARISON WITH $P_H8.3$

By substracting the figures in the first part of Table IV from the corresponding figures in the second part, the differences between the quantity of sodium hydroxid used in titrating to the hydrogen-ion concentration P_H7 and $P_H8.3$ are obtained. Also, by subtracting the figures

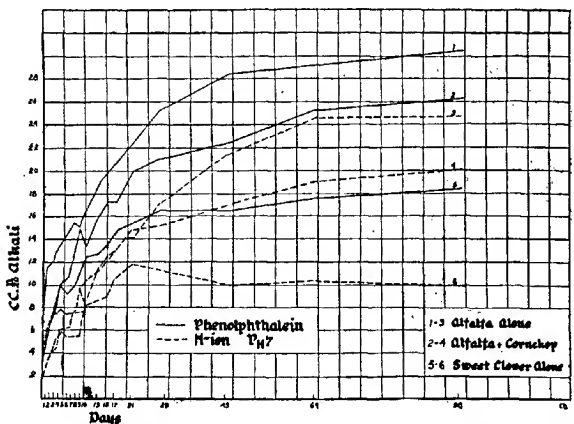

FIG. 3.—Graphs showing quantity, in cubic centimeters, of alkali used in alcoholic extract of silage: comparison of colorimetric titrations with phenolphthalein, and electrometric titrations to P_H7.

in the second part of Table IV from those in the third section the differences for the hydrogen-ion concentration $P_H8.3$ and $P_H9.3$ are obtained. The figures so obtained are found in Table V for both the water extract and the alcoholic extract.

The data in Table show that—

(1) The water extracts in comparison with the alcoholic extracts give substantially the same results (fig. 3–5).

(2) There is no significant change in the differences in the number of cubic centimeters of *N/20* sodium hydroxid used in titrating to a concentration, $P_H8.3$, in comparison with P_H7, as the silage matures—that is, the difference between the two points tends to be constant.

[1] SWANSON, C. O., and TAGUE, E. L. *op. cit.*, p. 284.

TABLE V.—*Differences in the quantity, in cubic centimeters, of N/20 sodium hydroxid used to titrate to P_H 8.3 in comparison with P_H 7; also between P_H 9.3 and P_H 8.3*

FOR WATER EXTRACT OF SILAGE

Age of silage.	Alfalfa alone.		Sweet clover alone.		Sweet clover and cornchop.	
	Increase from P_H7 to P_H8.3.	Increase from P_H8.3 to P_H9.3.	Increase from P_H7 to P_H8.3.	Increase from P_H8.3 to P_H9.3.	Increase from P_H7 to P_H8.3.	Increase from P_H8.3 to P_H9.3.
Days.	*A*	*B*	*A*	*B*	*A*	*B*
0	1.0	1.8	1.9	3.4	1.7	1.9
2	2.6	3.6	2.4	6.2	3.3	4.7
2	4.7	7.8	2.1	5.5	2.4	5.3
3	5.0	7.9	3.5	5.4	3.7	5.1
4	3.6	6.6	3.5	7.0	3.5	3.7
6	4.4	6.7	2.7	4.8	3.0	5.4
7	4.6	5.4				
8	3.7	5.9	2.3	5.0	2.8	5.7
10	4.1	8.9	2.5	5.2	3.0	5.4
13	3.5	7.8	2.4	5.5	2.6	7.7
15	3.0	8.7	2.9	7.8	2.7	9.8
17	3.1	12.8	2.7	6.8	2.7	9.6
21	6.5	10.2	3.3	7.5	4.6	8.9
29	5.8	11.7	3.0	9.6	3.5	7.5
43	7.0	10.1	3.8	7.0	4.0	7.3
64	7.5	11.0	3.5	7.1	4.9	7.1
98	4.5	12.6	1.6	6.8	4.5	5.1

FOR ALCOHOLIC EXTRACT OF SILAGE

0	2.1	3.1	1.3	2.0	1.1	1.9
1	3.2	5.8	1.7	3.6	2.4	3.6
2	3.5	6.3	2.2	4.4	1.8	4.5
3	3.3	6.9	2.2	4.7	2.4	5.2
4	3.4	7.2	2.4	6.0	2.2	5.6
6	3.8	7.9	2.3	5.1	2.6	6.2
7	3.6	7.8				
8	3.6	10.3	2.3	5.7	2.8	5.8
10	3.6	9.2	2.8	6.3	2.7	6.6
13	3.7	9.5	2.8	6.0	2.1	7.2
15	3.8	9.2	2.7	6.7	2.6	6.4
17	3.8	10.3	3.0	7.2	2.6	7.6
21	3.9	10.1	3.2	7.4	3.1	7.8
29	3.5	11.9	3.1	8.3	3.1	8.5
43	4.4	12.3	2.9	8.1	3.4	7.9
64	3.8	12.4	3.0	8.8	4.0	8.8
98	4.5	13.3	2.9	8.5	3.6	9.0

(3) The quantity of *N/20* sodium hydroxid used in titrating to a concentration, P_H9.3, in comparison with the concentration, P_H8.3, increases as the silage matures. This increase is most notable in the silage from alfalfa alone. This larger and larger neutralization or absorption of the sodium hydroxid as the silage becomes older is probably due to the production of substances of the nature of proteoses or peptones. The proteins undergo a splitting process. When the *N/20* sodium hydroxid is

added, it combines with these hydrolytic protein products in larger and larger amounts as the hydrogen-ion concentration is reduced below $P_H 8.3$ and more so when it approaches 9.3. If the titrations had been

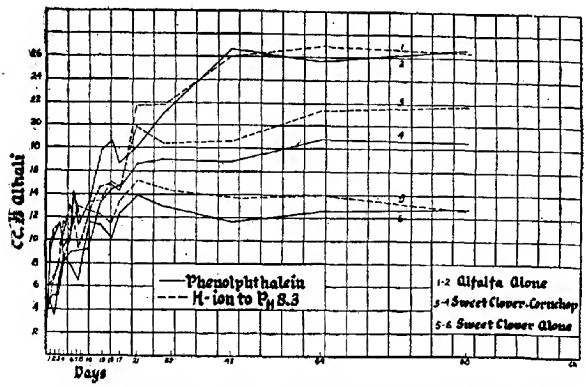

FIG. 4.—Graphs showing quantity, in cubic centimeters, of alkali used in water extract of silage; comparison of colorimetric titrations with phenolphthalein, and electrometric titrations to $P_H 8.3$.

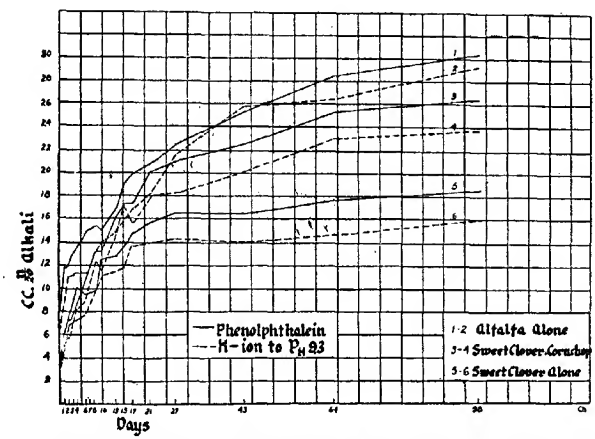

FIG. 5.—Graphs showing quantity, in cubic centimeters, of alkali used in alcoholic extract of silage; comparison of colorimetric titrations, with phenolphthalein, and electrometric titrations to $P_H 9.3$.

carried to a concentration of $P_H 10$ or lower, the differences obtained might have been even greater. Some work done in our laboratory on the hydrolysis of wheat substantiates this supposition.

(4) The fact that sweet clover shows smaller differences than alfalfa in these respects indicates that this protein hydrolysis takes place to a less extent in sweet clover than in alfalfa. This would mean that under practical conditions good silage is more readily made from sweet clover than from alfalfa. The addition of corn meal to sweet clover did not seem to have any distinct advantage. Silage from sweet clover alone was as good as that made from sweet clover and corn meal. With alfalfa the addition of corn meal was an advantage, as shown in our previous work.[1]

NITROGEN IN AMINO (NH_2) FORM

TITRATIONS WITH THE HYDROGEN ELECTRODE

It was stated that when making the determinations for acidity the hydrogen-ion concentration had been reduced to $P_H9.3$, or the theoretical points of color change for thymolphthalein, 25 cc. of the formaldehyde solution was added and the titration resumed till the hydrogen-ion concentration was again $P_H9.3$. This second titration should give figures for calculating the nitrogen in amino form. Whether this is the case is a question which we do not attempt to answer in the present paper. According to Sörensen,[2] who first elaborated the method, the titrations should first be carried to the neutral point for phenolphthalein, or $P_H8.3$, then the formaldehyde solution added, and the titration repeated till the point of color change for thymolphthalein, or $P_H9.3$ is reached. The number of cubic centimeters of *N/20* sodium hydroxid used in this second titration multiplied by 0.7 give the number of milligrams of titrable nitrogen in the mixture. If this is correct, the results of the electrometric titrations as we made them are too low for amino nitrogen when the number of cubic centimeters obtained in this last titration are multiplied by 0.7. But as both the $P_H8.3$ and the $P_H9.3$ points were determined in the titrations for acidity, a correction can be introduced. This is done in Table VI. In the A columns are given the number of cubic centimeters used in the second titration after the formalin (*HCHO*) was added to bring the P_H value again to the 9.3 point. In Table V is given the differences in titrations between titrating to $P_H8.3$ and $P_H9.3$. By adding these differences to the figures in the A columns of Table VI, the figures given in the B columns are obtained. These figures should be the same as if the titrations had been first carried to the $P_H8.3$ point in the acidity titration and then formalin added and then the titration resumed till the $P_H9.3$ point was reached. That this assumption is correct is substantiated by some work done by us on the changes which take place in

[1] SWANSON, C. O., and TAGUE, E. L. OP. CIT.

[2] SÖRENSEN, S. P. L. ENZYMSTUDIEN. *In* Biochem. Ztschr., Bd. 7, Heft ¼, p. 45–101. 1907. Allen's Commercial Organic Analysis . . . ed. 4, v. 8, p. 478, 488. Philadelphia. 1913.

the hydrolysis of wheat. By multiplying these figures in the B columns by 0.7 the number of milligrams of amino nitrogen are obtained.

TABLE VI.—*Quantity of nitrogen in titrable form as obtained by electrometric titration*

FOR WATER EXTRACT

Age of silage.	Alfalfa alone.			Sweet clover alone.			Sweet clover and cornmeal.		
	N/20 sodium hydroxid after adding formalin (cc.).	Quantity in A+column B, Table V, 2d sect. (cc.).	Mgm. amino nitrogen or cc. in B×0.7.	N/20 sodium hydroxid after adding formalin (cc.).	Quantity in A+column B, Table V, 2d sect. (cc.).	Mgm. amino nitrogen or cc. in B×0.7.	N/20 sodium hydroxid after adding formalin (cc.).	Quantity in A+column B, Table V, 2d sect. (cc.).	Mgm. amino nitrogen or cc. in B×0.7.
Days.	*A.*	*B*		*A*	*B*		*A*	*B*	
0	3.5	5.3	3.7	2.1	5.5	3.9	1.8	3.7	2.6
1	13.8	17.4	12.2	7.5	13.7	9.6	6.0	10.7	7.5
2	13.3	21.1	14.8	8.8	14.3	10.0	8.6	11.9	8.3
3	14.8	22.7	15.9	10.7	16.1	11.3	9.1	14.2	9.9
4	16.0	22.6	15.8	13.3	20.3	14.2	11.8	15.5	10.9
6	18.5	25.2	17.6	14.0	18.8	13.2	13.9	19.5	13.5
7	22.0	27.4	19.2	.....	.....	.....	.....	.....	.....
8	22.2	28.1	19.7	12.0	17.0	11.9	13.0	18.7	113.1
10	22.2	31.1	21.8	16.4	21.6	15.1	13.5	18.9	13.2
13	23.2	31.0	21.7	17.6	23.1	16.2	18.1	25.8	18.1
15	23.9	32.6	22.8	15.2	23.0	16.1	17.1	26.9	18.8
17	24.0	36.8	25.8	14.8	21.6	15.1	16.8	26.4	18.5
21	28.8	39.0	27.3	19.2	26.7	18.7	17.0	27.9	19.5
29	28.1	39.8	27.9	19.1	28.7	21.1	19.1	26.6	18.6
43	28.3	38.4	16.9	16.5	23.5	16.5	15.4	22.7	15.9
64	27.7	38.7	27.1	16.5	23.6	16.5	14.1	21.2	14.8
98	26.8	39.4	27.6	18.1	24.9	17.4	15.5	20.6	14.4

FOR ALCOHOLIC EXTRACT

0	3.1	6.2	4.3	2.0	4.0	2.8	2.0	3.9	2.7
1	9.0	14.8	10.4	5.0	8.6	6.0	4.7	8.3	5.8
2	13.1	19.4	13.6	6.4	10.8	7.6	6.2	10.7	7.5
3	12.9	19.8	13.9	7.8	12.5	8.8	6.4	11.6	8.1
4	12.5	19.7	13.8	9.3	15.7	11.0	8.8	14.4	10.1
6	14.1	22.0	15.4	8.3	13.4	9.4	8.2	14.4	10.1
7	13.3	21.1	14.8	.....	.....	.....	.....	.....	.....
8	14.8	25.1	17.6	9.2	14.9	10.4	10.1	15.9	11.1
10	13.8	23.0	16.1	10.3	16.0	11.6	9.6	16.2	11.3
13	16.5	26.0	18.2	11.6	17.6	12.3	11.4	18.6	13.0
15	16.7	25.9	18.1	12.0	18.7	13.1	13.2	19.6	13.7
17	17.4	27.7	19.4	12.0	19.2	13.4	12.6	20.2	14.1
21	16.5	26.6	18.6	12.6	20.0	14.0	14.2	22.0	15.4
29	19.3	21.2	21.8	13.5	21.8	15.3	13.8	22.3	15.6
43	22.6	34.9	24.4	13.2	21.3	14.9	14.3	22.2	15.5
64	23.0	35.4	24.8	14.6	23.4	16.4	13.8	22.6	15.8
98	23.0	36.3	25.4	16.1	14.6	17.2	16.0	25.0	17.5

The data in Table VI show that—

(1) The amount of amino nitrogen in the alcoholic extract and in the water extract is practically the same for the matured silage. In silage from 1 to about 2 weeks old the water extract shows the presence of a larger amount of amino nitrogen.

(2) The amount of amino nitrogen in the silage made from alfalfa is notably larger than that in the silage made from sweet clover.

(3) The amount of amino nitrogen in the silage made from sweet clover alone is practically the same as that in the silage made from sweet clover and corn meal. The addition of corn meal does not have any apparent influence on the amount of amino nitrogen produced.

TITRATIONS WITH INDICATORS

Titrations with indicators were made as follows: Twenty-five cc. of the extract were pipetted into a flask with 200 cc. of carbon-dioxid-free water, and 25 cc. of formalin added (1 part of 40 per cent formalin to 2 parts of carbon-dioxid-free water). This had been made neutral to thymolphthalein with sodium hydroxid. The mixture was well shaken and allowed to stand for 15 minutes, when it was titrated to a distant blue with *N*/20 sodium hydroxid, using 5 cc. of thymolphthalein as indicator. The total number of cubic centimeters obtained in this titration less the number of cubic centimeters used to obtain the acidity to phenolphthalein represents the number of cubic centimeters obtained in this titration less the number of cubic centimeters used to obtain the acidity to phenolphthalein represents the number of cubic centimeters due the titrable nitrogen. The figures as obtained are given in the A column of Table VII.

From the figures given in Table VII must be subtracted the figures for the acidity titration given in Table III. The remainder represents the amount of acidity due to the titrable nitrogen. These differences are given in the B columns of Table VII. These differences multiplied by 0.7 give the weight of titrable nitrogen. The results are given in the C columns. A comparison of the results in Table VI with those in VII shows that the results obtained by the electrometric and colorimetric methods are essentially the same. A direct comparison between the results on the water and the alcoholic extract, colorimetric titration, is given in Table VIII.

TABLE VII.—*Quantity of nitrogen in titrable form as obtained by colorimetric titration*

FOR WATER EXTRACT

Age of silage.	Alfalfa alone.			Sweet clover alone.			Sweet clover and corn meal.		
	Cc. of N/20 sodium hydroxid corrected for acidity to —		Mgm. of titrable nitrogen in BXo.7.	Cc. of N/20 sodium hydroxid corrected for acidity to —		Mgm. of titrable nitrogen in BXo.7.	Cc. of N/20 sodium hydroxid corrected for acidity to —		Mgm. of titrable nitrogen in BXo.7.
	Thymol.	Phenol.		Thymol.	Phenol.		Thymol.	Phenol.	
Days.	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	5.7	2.4	1.7	6.4	3.6	2.5	5.2	1.9	1.3
1	14.9	5.9	4.1	18.2	13.6	9.5	15.1	9.8	6.9
2	28.2	17.8	12.5	20.4	15.0	10.5	17.5	12.0	8.4
3	29.8	18.4	12.9	23.8	17.5	12.3	19.1	13.5	9.5
4	27.6	18.5	13.0	26.0	17.3	12.1	23.0	14.8	10.4
6	29.1	18.5	13.0	21.7	13.7	9.6	24.7	15.7	11.0
7	34.0	19.6	13.7	.....	.....	.....	.....	.....	.....
8	35.4	23.9	16.7	22.6	16.2	11.3	27.0	13.8	9.7
10	37.5	24.1	16.9	29.3	17.7	12.4	27.6	18.3	12.8
13	35.5	17.7	12.4	28.7	17.4	12.2	35.1	21.9	15.3
15	40.0	21.5	15.1	31.5	21.2	12.8	33.3	19.0	13.3
17	46.9	30.3	21.2	30.5	18.3	12.8	35.1	20.3	14.2
21	52.7	34.6	24.2	40.8	27.0	18.9	39.9	23.3	16.3
29	53.3	32.5	22.8	40.5	27.5	19.3	45.8	28.8	20.2
43	56.2	29.6	20.7	35.5	23.8	16.7	40.2	23.4	16.4
64	60.2	34.5	24.2	36.4	23.8	16.7	47.0	28.1	19.7
98	64.7	38.0	26.6	39.5	26.7	18.7	45.8	27.2	19.0

FOR ALCOHOLIC EXTRACT

0	14.1	6.4	4.5	7.7	4.1	2.9	8.2	4.4	3.1
1	27.2	15.5	10.9	14.3	8.2	5.7	14.6	8.9	6.2
2	31.4	19.4	13.6	18.5	11.3	7.9	17.3	10.4	7.3
3	32.5	19.6	13.7	20.9	12.2	8.5	17.8	10.2	7.1
4	32.0	18.5	13.0	24.9	14.8	10.4	22.8	12.8	9.0
6	34.7	19.8	13.9	21.9	12.5	8.8	24.8	13.9	9.7
7	36.0	20.6	14.4	.....	.....	.....	.....	.....	.....
8	39.7	24.7	17.3	23.9	14.0	9.8	29.4	14.6	10.2
10	41.5	24.6	17.2	27.0	14.5	10.2	27.2	14.0	9.8
13	44.2	25.2	17.7	29.7	16.9	11.8	32.6	16.5	11.6
15	45.5	25.6	17.9	30.6	17.0	11.9	33.9	16.6	11.6
17	46.9	26.1	18.3	33.4	18.7	13.1	36.0	18.7	13.1
21	47.9	25.5	17.9	34.0	18.5	13.0	40.7	20.7	14.5
29	55.7	30.5	21.4	35.2	18.8	13.2	41.1	20.1	14.1
43	60.0	31.6	22.1	38.7	22.2	15.5	44.7	22.3	15.6
64	64.0	33.6	23.5	39.1	21.4	15.0	44.4	19.2	13.4
98	.....	.....	.....	42.0	23.5	16.5	49.3	23.0	16.1

A comparison of the amount of amino nitrogen obtained by the Van Slyke method with the amounts obtained by the electrometric and colorimetric titrations was made. For this comparison bottles of matured alfalfa silage and sweet-clover silage were used. The Van Slyke determination was made in the usual way. The titrable nitrogen

was determined both by the electrometric and the colorimetric methods. The results from the Van Slyke method were very much larger, but we did not investigate the reasons for this. To make a thorough comparison of the Van Slyke method with the formol-titration method would probably require as extended an investigation as the one reported in this paper.

TABLE VIII.—*Comparison of amount of titrable nitrogen stated as milligrams per 5 gm. of silage in alcoholic and water extracts, colorimetric titration*

Age of silage.	Alfalfa alone.		Sweet clover alone.		Sweet clover and corn meal.	
	Water.	Alcohol.	Water.	Alcohol.	Water.	Alcohol.
Days.						
0	1.7	4.5	2.5	2.9	1.3	3.1
1	4.1	10.9	9.5	5.7	6.9	6.2
2	12.5	13.6	10.5	7.9	8.4	7.3
3	12.9	13.7	12.3	8.5	9.5	7.1
4	13.0	13.0	12.1	10.4	10.4	9.0
6	13.0	13.9	9.6	8.8	11.0	9.7
7	13.7	14.4	.....	.....	.....	.....
8	16.7	17.3	11.3	9.8	9.7	10.2
10	16.9	17.2	12.4	10.2	12.8	9.8
13	12.4	17.6	12.2	11.8	15.3	11.6
15	15.1	17.9	12.8	11.9	13.3	11.6
17	21.2	18.3	12.8	13.1	14.2	13.1
21	24.2	17.9	18.9	13.0	16.3	14.5
29	22.8	21.4	19.3	13.2	20.2	14.1
43	20.7	22.1	16.7	15.5	16.4	15.6
64	24.2	23.5	16.7	15.0	19.7	13.4
98	26.6	.....	18.7	16.5	19.0	16.1

TOTAL, STUTZER'S, OR ALBUMINOID, AND AMID NITROGEN IN SILAGE, KJELDAHL METHOD

The total and Stutzer's nitrogen, sometimes called "albuminoid nitrogen," were determined on the samples from the moisture determinations. The result stated as milligrams per 5 gm. of silage are given in Table IX.

The results given in Table IX show that—

(1) In silage from alfalfa alone the nitrogen in amid [1] form is approximately one-half of the total. In silage from sweet clover alone it is a little more than one-half of the total, and when corn meal is added, the proportion is somewhat smaller.

[1] The term "amid" is used simply to designate the difference between the total and albuminoid nitrogen as determined by Stutzer's method (WILEY, H. W., ED. OFFICIAL AND PROVISIONAL METHODS OF ANALYSIS, ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS, AS COMPILED BY THE COMMITTEE ON REVISION OF METHODS. U. S. Dept. Agri. Bur. Chem. Bul. 107 (rev.), p. 38. 1908. Reprinted, 1912.

(2) The nitrogen in amid form as determined by this method does not show the same degree of progressive increase as the nitrogen determined by titration, as shown in Table VIII. The drying of the sample may have something to do with this. That the process of drying may influence the form of nitrogen in forage material is shown by investigations now in progress at this laboratory.

(3) The amount of nitrogen in amid form in matured silage is a little larger as determined by Stutzer's method than by the formaldehyde titration method, but the differences are not large. Compare Tables VI and VII with Table IX.

TABLE IX.—*Total nitrogen, Stutzer's nitrogen, and amid nitrogen in silage*

[Results are expressed as milligrams per 5 gms. of silage.]

Age of silage.	Alfalfa alone.			Sweet clover alone.			Sweet clover and corn meal.		
	Total nitrogen.	Stutzer's nitrogen.	Amid nitrogen.	Total nitrogen.	Stutzer's nitrogen.	Amid nitrogen.	Total nitrogen.	Stutzer's nitrogen.	Amid nitrogen.
Days.									
1	58.0	29.0	29.0	33.5	21.5	12.0	42.0	27.0	15.0
2	56.5	29.5	27.0	36.0	22.0	14.0	41.5	26.5	15.0
3	60.0	32.0	28.0	36.5	20.0	16.5	40.0	25.5	14.5
4	58.5	29.0	29.5	36.5	20.0	16.5	35.0	24.5	10.5
6	70.5	25.0	45.5	35.5	19.5	16.0	43.0	26.0	17.0
7	52.5	26.0	26.5	.....	.....	.....	.....	.....	.....
8	49.5	26.5	23.0	35.0	19.0	16.0	38.0	23.5	14.5
10	52.0	25.5	26.5	33.5	16.5	17.0	39.5	23.5	16.0
13	52.0	24.5	27.5	32.5	15.5	17.0	42.5	24.0	18.5
15	48.5	22.5	26.0	30.0	15.0	15.0	41.5	23.0	18.5
17	52.0	24.0	28.0	37.0	16.5	20.5	40.5	21.5	19.0
21	53.5	27.5	26.0	36.0	15.0	21.0	41.0	22.0	19.0
29	59.0	26.5	32.5	34.0	14.0	20.0	39.0	21.5	17.5
43	54.5	30.0	24.5	34.0	16.0	18.0	35.5	19.5	16.0
64	54.0	25.5	28.5	29.5	14.0	15.5	35.5	20.0	15.5
98	51.0	24.0	27.0	32.0	14.0	18.0	37.0	19.0	18.0

NITROGEN IN WATER AND ALCOHOLIC EXTRACTS OF SILAGE, KJELDAHL METHOD

The water and alcoholic extracts of silage were used for the determination of nitrogen, Kjeldahl method, both total and that not precipitated by phosphotungstic acid. The results stated in milligrams per 5 grams of silage are given in Table X.

The results given in Table X show that—

(1) The water and the alcoholic extracts gave practically the same amount of total nitrogen, showing that the solvent action of 50 per sent alcohol is not different from that of water.

(2) The soluble nitrogen is approximately two-thirds of the total in the silage made from alfalfa alone and from sweet clover alone. When corn meal was added to sweet clover, the proportion of water-soluble to total nitrogen was a little more than one-half. Compare Table IX with Table X.

(3) In the water extract the nitrogen not precipitated by phosphotungstic acid is approximately three-fourths of the total soluble nitrogen. In the alcoholic extract the nitrogen precipitated by phosphotungstic acid is practically the same in the amount as the total soluble in 50 per cent alcohol.

(4) Thus, while the total solubility is not much different in the water and alcoholic extracts, the nature of the solution is different as measured by the precipitating action of phosphotungstic acid. This is either due to the solubility of the protein phosphotungstate in the alcohol or to the fact that the ionization is such that the precipitate will not form when the phosphotungstic acid is added.

TABLE X.—*Total nitrogen and nitrogen not precipitated by phosphotungstic acid in water and alcoholic extracts of silage*

[Results expressed as milligrams of nitrogen from 5 gm. of silage.]

Age of silage.	Alfalfa alone.				Sweet clover alone.				Sweet clover and corn meal.			
	Water extract.		Alcoholic extract.		Water extract.		Alcoholic extract.		Water extract.		Alcoholic extract.	
	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.	Total nitrogen.	Nitrogen not precipitated by phosphotungstic acid.
Days.												
0	8.5	3.0	.....	.....	7.0	4.5	.....	.....	11.0	4.5	.....	.....
1	26.0	15.0	23.0	24.5	19.0	10.5	15.5	14.5	17.0	9.0	16.5	14.5
2	30.0	20.5	26.0	28.0	7.5	12.0	15.0	16.5	16.0	11.0	17.0	15.5
3	29.0	20.0	28.0	29.5	19.0	12.0	18.0	18.5	18.0	10.5	18.0	16.0
4	27.5	16.5	26.5	28.5	20.0	14.5	20.0	20.5	17.0	12.0	19.0	19.0
6	26.5	21.0	29.0	30.5	18.5	13.5	17.5	18.0	20.0	15.0	21.5	19.0
7	30.0	24.0	26.0	28.0	.....	.....	.....	.....	.....	.....	.....	.....
8	30.5	19.0	29.5	32.0	15.0	14.0	18.0	19.0	19.5	14.0	21.5	19.5
10	30.5	23.5	30.0	32.0	22.0	17.5	20.0	20.5	20.5	14.5	22.0	20.0
13	29.0	22.0	31.0	34.0	21.0	15.5	21.5	21.0	22.0	16.0	24.5	22.5
15	31.0	22.5	30.0	31.0	21.0	17.5	20.5	21.0	22.5	17.0	24.5	22.5
17	34.0	31.5	31.5	32.5	31.5	26.5	21.0	21.5	21.5	16.5	24.5	22.5
21	33.5	24.5	31.0	32.0	22.5	16.0	22.0	22.5	23.5	16.0	26.0	25.0
29	35.0	24.5	35.0	36.5	23.0	17.5	22.5	24.0	25.0	18.0	26.0	24.0
43	38.0	27.0	36.5	31.5	22.5	16.0	21.5	23.0	23.5	17.5	25.5	24.5
64	37.0	28.5	38.0	42.0	21.0	14.5	22.5	26.5	23.0	17.0	14.0	26.5
98	34.5	27.5	30.5	38.0	20.5	12.5	25.0	24.0	24.0	19.5	27.5	26.5

SUMMARY

(1) In this paper have been presented the results of making determinations both by the colorimetric and electrometric methods in the water and alcoholic extracts of silage made from alfalfa alone, from sweet clover alone, and from sweet clover plus corn meal. These extracts were also used for the determinations of nitrogen in amino form by the colorimetric and electrometric methods. Total nitrogen and albuminoid, or Stutzer's nitrogen, were also determined on these extracts. Quart milk bottles were used as containers for the silage. A number of bottles of each kind of silage were made and these were opened at increasingly longer intervals of time. In this way the progressive chemical changes were traced.

(2) The weight of the bottles just after filling and when opened showed that the losses were approximately 1 per cent.

(3) The acidity of the alcoholic extracts of the three kinds of silage was greater than that of the water extract when the titration was made to the point of color change for phenolphthalein. When the electrometric method was used and the titration was made to a hydrogen-ion concentration of $P_H 8.3$, the point of color change for phenolphthalein, there was no significant difference between the results obtained on the water extract and that of the alcoholic extract. The greater values obtained on the alcoholic extracts with the colorimetric method are probably due to the highly colored material extracted by the alcohol. These mask the end point.

(4) Most of the acidity is developed in the first 15 days.

(5) Adding corn meal to sweet clover increases the amount of acidity in the resulting silage.

(6) The amount of amino nitrogen is practically the same in the water and the alcoholic extracts. The amount of amino nitrogen in silage made from alfalfa alone is notably larger than that made from sweet clover alone. The addition of corn meal to sweet clover has no influence on the amount of amino nitrogen developed.

(7) The amount of nitrogen in amid form as determined by Stutzer's method was a little larger than the amount of nitrogen in amino form as determined by the formaldehyde method, but the differences were not large. The nitrogen in amid form was approximately one-half of the total.

(8) Approximately two-thirds of the total nitrogen in silage is soluble in water and 50 per cent alcohol, the solvent action of the two being nearly the same.

(9) From the various data presented, it appears that silage can be made from sweet clover alone with less difficulty than from alfalfa alone.